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(54) Massive parallel method for decoding DNA and RNA

Massives Parallelverfahren zur Dekodierung von DNA und RNA

Méthode massive d'analyse parallèle destinée à décoder l'ADN et l'ARN

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- **METZKER M L ET AL: "TERMINATION OF DNA SYNTHESIS BY NOVEL 3'-MODIFIED-DEOXYRIBONUCLEOSIDE 5'-TRIPHOSPHATES" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 22, no. 20, 1994, pages 4259-4267, XP001068669 ISSN: 0305-1048**
- **HULTMAN T ET AL: "DIRECT SOLID PHASE SEQUENCING OF GENOMIC AND PLASMID DNA USING MAGNETIC BEADS AS SOLID SUPPORT" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 17, no. 13, 11 July 1989 (1989-07-11), pages 4937-4946, XP000371656 ISSN: 0305-1048**
- **LEE ET AL.: 'New energy transfer dyes for DNA sequencing' NUCLEIC ACIDS RESEARCH vol. 25, no. 14, 1997, pages 2816 - 2822, XP002909904**
- **ROSENBLUM ET AL.: 'New dye-labeled terminators for improved DNA sequencing patterns' NUCLEIC ACIDS RESEARCH vol. 25, no. 22, 1997, pages 4500 - 4504, XP002909905**

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EP 1 337 541 B1

Description

[0001] This application claims the benefit of U.S. Provisional Application No. 60/300,894, filed June 26, 2001, and is a continuation-in-part of U.S. Serial No. 09/684,670, filed October 6, 2000.

Background Of The Invention

[0002] Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

[0003] The ability to sequence deoxyribonucleic acid (DNA) accurately and rapidly is revolutionizing biology and medicine. The confluence of the massive Human Genome Project is driving an exponential growth in the development of high throughput genetic analysis technologies. This rapid technological development involving chemistry, engineering, biology, and computer science makes it possible to move from studying single genes at a time to analyzing and comparing entire genomes.

[0004] With the completion of the first entire human genome sequence map, many areas in the genome that are highly polymorphic in both exons and introns will be known. The pharmacogenomics challenge is to comprehensively identify the genes and functional polymorphisms associated with the variability in drug response (Roses, 2000). Resequencing of polymorphic areas in the genome that are linked to disease development will contribute greatly to the understanding of diseases, such as cancer, and therapeutic development. Thus, high-throughput accurate methods for resequencing the highly variable intron/exon regions of the genome are needed in order to explore the full potential of the complete human genome sequence map. The current state-of-the-art technology for high throughput DNA sequencing, such as used for the Human Genome Project (Pennisi 2000), is capillary array DNA sequencers using laser induced fluorescence detection (Smith et al., 1986; Ju et al. 1995, 1996; Kheterpal et al. 1996; Salas-Solano et al. 1998). Improvements in the polymerase that lead to uniform termination efficiency and the introduction of thermostable polymerases have also significantly improved the quality of sequencing data (Tabor and Richardson, 1987, 1995). Although capillary array DNA sequencing technology to some extent addresses the throughput and read length requirements of large scale DNA sequencing projects, the throughput and accuracy required for mutation studies needs to be improved for a wide variety of applications ranging from disease gene discovery to forensic identification. For example, electrophoresis based DNA sequencing methods have difficulty detecting heterozygotes unambiguously and are not 100% accurate in regions rich in nucleotides comprising guanine or cytosine due to compressions (Bowling et al. 1991; Yamakawa et al. 1997). In addition, the first few bases after the priming site are often masked by the high fluorescence signal from excess dye-labeled primers or dye-labeled terminators, and are therefore difficult to identify. Therefore, the requirement of electrophoresis for DNA sequencing is still the bottleneck for high-throughput DNA sequencing and mutation detection projects.

[0005] The concept of sequencing DNA by synthesis without using electrophoresis was first revealed in 1988 (Hyman, 1988) and involves detecting the identity of each nucleotide as it is incorporated into the growing strand of DNA in a polymerase reaction. Such a scheme coupled with the chip format and laser-induced fluorescent detection has the potential to markedly increase the throughput of DNA sequencing projects. Consequently, several groups have investigated such a system with an aim to construct an ultra high-throughput DNA sequencing procedure (Cheeseman 1994, Metzker et al. 1994). Thus far, no complete success of using such a system to unambiguously sequence DNA has been reported. The pyrosequencing approach that employs four natural nucleotides (comprising a base of adenine (A), cytosine (C), guanine (G), or thymine (T)) and several other enzymes for sequencing DNA by synthesis is now widely used for mutation detection (Ronaghi 1998). In this approach, the detection is based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to adenosine triphosphate (ATP) by sulfurylase, and the subsequent production of visible light by firefly luciferase. This procedure can only sequence up to 30 base pairs (bps) of nucleotide sequences, and each of the 4 nucleotides needs to be added separately and detected separately. Long stretches of the same bases cannot be identified unambiguously with the pyrosequencing method.

[0006] More recent work in the literature exploring DNA sequencing by a synthesis method is mostly focused on designing and synthesizing a photocleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of deoxynucleoside triphosphates (dNTPs) (Welch et al. 1999) (WO 00/53805, WO 91/06878, WO 01/92284). Limited success for the incorporation of the 3'-modified nucleotide by DNA polymerase is reported. The reason is that the 3'-position on the deoxyribose is very close to the amino acid residues in the active site of the polymerase, and the polymerase is therefore sensitive to modification in this area of the deoxyribose ring. On the other hand, it is known that modified DNA polymerases (Thermo Sequenase and Taq FS polymerase) are able to recognize nucleotides with extensive modifications with bulky groups such as energy transfer dyes at the 5-position of the pyrimidines (T and C) and at the 7-position of purines (G and A) (Rosenblum et al. 1997, Zhu et al. 1994). The ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP) have been determined (Pelletier et al.

1994) which supports this fact. As shown in Figure 1, the 3-D structure indicates that the surrounding area of the 3'-position of the deoxyribose ring in ddCTP is very crowded, while there is ample space for modification on the 5-position the cytidine base.

[0007] The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye or a mass tag through a cleavable linker to the nucleotide base or an analogue of the nucleotide base, such as to the 5-position of the pyrimidines (T and C) and to the 7-position of the purines (G and A), to use a small cleavable chemical moiety to cap the 3'-OH group of the deoxyribose to make it nonreactive, and to incorporate the nucleotide analogues into the growing DNA strand as terminators. Detection of the unique label will yield the sequence identity of the nucleotide. Upon removing the label and the 3'-OH capping group, the polymerase reaction will proceed to incorporate the next nucleotide analogue and detect the next base.

[0008] It is also desirable to use a photocleavable group to cap the 3'-OH group. However, a photocleavable group is generally bulky and thus the DNA polymerase will have difficulty to incorporate the nucleotide analogues containing a photocleavable moiety capping the 3'-OH group. If small chemical moieties that can be easily cleaved chemically with high yield can be used to cap the 3'-OH group, such nucleotide analogues should also be recognized as substrates for DNA polymerase. It has been reported that 3'-O-methoxy-deoxynucleotides are good substrates for several polymerases (Axelrod et al. 1978). 3'-O-allyl-dATP was also shown to be incorporated by Ventri(exo-) DNA polymerase in the growing strand of DNA (Metzker et al. 1994). However, the procedure to chemically cleave the methoxy group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methoxy group to cap the 3'-OH group for sequencing DNA by synthesis. An ester group was also explored to cap the 3'-OH group of the nucleotide, but it was shown to be cleaved by the nucleophiles in the active site in DNA polymerase (Canard et al. 1995). Chemical groups with electrophiles such as ketone groups are not suitable for protecting the 3'-OH of the nucleotide in enzymatic reactions due to the existence of strong nucleophiles in the polymerase. It is known that MOM (-CH₂OCH₃) and allyl (-CH₂CH=CH₂) groups can be used to cap an -OH group, and can be cleaved chemically with high yield (Ireland et al. 1986; Kamal et al. 1999). The approach disclosed in the present application is to incorporate nucleotide analogues, which are labeled with cleavable, unique labels such as fluorescent dyes or mass tags and where the 3'-OH is capped with a cleavable chemical moiety such as either a MOM group (-CH₂OCH₃) or an allyl group (-CH₂CH=CH₂), into the growing strand DNA as terminators. The optimized nucleotide set (3'-RO-A^{LABEL1}, 3'-RO-C^{LABEL2}, 3'-RO-G^{LABEL3}, 3'-RO-T^{LABEL4}, where R denotes the chemical group used to cap the 3'-OH) can then be used for DNA sequencing by the synthesis approach.

[0009] There are many advantages of using mass spectrometry (MS) to detect small and stable molecules. For example, the mass resolution can be as good as one dalton. Thus, compared to gel electrophoresis sequencing systems and the laser induced fluorescence detection approach which have overlapping fluorescence emission spectra, leading to heterozygote detection difficulty, the MS approach disclosed in this application produces very high resolution of sequencing data by detecting the cleaved small mass tags instead of the long DNA fragment. This method also produces extremely fast separation in the time scale of microseconds. The high resolution allows accurate digital mutation and heterozygote detection. Another advantage of sequencing with mass spectrometry by detecting the small mass tags is that the compressions associated with gel based systems are completely eliminated.

[0010] In order to maintain a continuous hybridized primer extension product with the template DNA, a primer that contains a stable loop to form an entity capable of self-priming in a polymerase reaction can be ligated to the 3' end of each single stranded DNA template that is immobilized on a solid surface such as a chip. This approach will solve the problem of washing off the growing extension products in each cycle.

[0011] Saxon and Bertozzi (2000) developed an elegant and highly specific coupling chemistry linking a specific group that contains a phosphine moiety to an azido group on the surface of a biological cell. In the present application, this coupling chemistry is adopted to create a solid surface which is coated with a covalently linked phosphine moiety, and to generate polymerase chain reaction (PCR) products that contain an azido group at the 5' end for specific coupling of the DNA template with the solid surface. One example of a solid surface is glass channels which have an inner wall with an uneven or porous surface to increase the surface area. Another example is a chip.

[0012] The present application discloses a novel and advantageous system for DNA sequencing by the synthesis approach which employs a stable DNA template, which is able to self prime for the polymerase reaction, covalently linked to a solid surface such as a chip, and 4 unique nucleotides analogues (3'-RO-A^{LABEL1}, 3'-RO-C^{LABEL2}, 3'-RO-G^{LABEL3}, 3'-RO-T^{LABEL4}). The success of this novel system will allow the development of an ultra high-throughput and high fidelity DNA sequencing system for polymorphism, pharmacogenetics applications and for whole genome sequencing. This fast and accurate DNA resequencing system is needed in such fields as detection of single nucleotide polymorphisms (SNPs) (Chee et al. 1996), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), identification in forensics, and genetic disease association studies.

Summary Of The Invention

[0013] This invention is directed to a method for sequencing a nucleic acid by sequentially determining the identity of

a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) determining the identity of the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the -OH group at the 3' -position of the deoxyribose to uncap the -OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (viii) so as to determine, for each repetition, the identity of the newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and

wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

[0014] The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

[0015] The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

Brief Description Of The Figures

[0016]

- 5 **Figure 1:** The 3D structure of the ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP). The left side of the illustration shows the mechanism for the addition of ddCTP and the right side of the illustration shows the active site of the polymerase. Note that the 3' position of the dideoxyribose ring is very crowded, while ample space is available at the 5' position of the cytidine base.
- 10 **Figure 2A-2B:** Scheme of sequencing by the synthesis approach. A: Example where the unique labels are dyes and the solid surface is a chip. B: Example where the unique labels are mass tags and the solid surface is channels etched into a glass chip. A, C, G, T; nucleotide triphosphates comprising bases adenine, cytosine, guanine, and thymine; d, deoxy; dd, dideoxy; R, cleavable chemical group used to cap the -OH group; Y, cleavable linker.
- 15 **Figure 3:** The synthetic scheme for the immobilization of an azido (N_3) labeled DNA fragment to a solid surface coated with a triarylphosphine moiety. Me, methyl group; P, phosphorus; Ph, phenyl.
- Figure 4:** The synthesis of triarylphosphine N-hydroxysuccinimide (NHS) ester.
- 20 **Figure 5:** The synthetic scheme for attaching an azido (N_3) group through a linker to the 5' end of a DNA fragment, which is then used to couple with the triarylphosphine moiety on a solid surface. DMSO, dimethylsulfonyl oxide.
- Figure 6A-6B:** Ligate the looped primer (B) to the immobilized single stranded DNA template forming a self primed DNA template moiety on a solid surface. P (in circle), phosphate.
- 25 **Figure 7:** Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique fluorescent dye attached to the base through a photocleavable linker and the 3'-OH is either exposed or capped with a MOM group or an allyl group. FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine. R = H, CH_2OCH_3 (MOM) or $CH_2CH=CH_2$ (Allyl).
- 30 **Figure 8:** A representative scheme for the synthesis of the nucleotide analogue $3'-RO-G-Tam$. A similar scheme can be used to create the other three modified nucleotides: $3'-RO-A-Dye1$, $3'-RO-C-Dye2$, $3'-RO-T-Dye4$. (i) tetrakis(triphenylphosphine)palladium(0); (ii) $POCl_3$, Bn_4N^+ pyrophosphate; (iii) NH_4OH ; (iv) $Na_2CO_3/NaHCO_3$ (pH = 9.0)/DMSO.
- 35 **Figure 9:** A scheme for testing the sequencing by synthesis approach. Each nucleotide, modified by the attachment of a unique fluorescent dye, is added one by one, based on the complimentary template. The dye is detected and cleaved to test the approach. Dye1 = Fam; Dye2 = R6G; Dye3 = Tam; Dye4 = Rox.
- 40 **Figure 10:** The expected photocleavage products of DNA containing a photo-cleavable dye (Tam). Light absorption (300 - 360 nm) by the aromatic 2-nitrobenzyl moiety causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond located in the 2-position followed by cleavage and decarboxylation (Pillai 1980).
- 45 **Figure 11:** Synthesis of **PC-LC-Biotin-FAM** to evaluate the photolysis efficiency of the fluorophore coupled with the photocleavable linker 2-nitrobenzyl group.
- Figure 12:** Fluorescence spectra (λ_{ex} = 480 nm) of **PC-LC-Biotin-FAM** immobilized on a microscope glass slide coated with streptavidin (a); after 10 min photolysis (λ_{irr} = 350 nm; ~ 0.5 mW/cm²) (b); and after washing with water to remove the photocleaved dye (c).
- 50 **Figure 13A-13B:** Synthetic scheme for capping the 3'-OH of nucleotide.
- Figure 14:** Chemical cleavage of the MOM group (top row) and the allyl group (bottom row) to free the 3'-OH in the nucleotide. CITMS = chlorotrimethylsilane.
- 55 **Figure 15A-15B:** Examples of energy transfer coupled dye systems, where Fam or Cy2 is employed as a light absorber (energy transfer donor) and Cl_2Fam , Cl_2R6G , Cl_2Tam , or Cl_2Rox as an energy transfer acceptor. Cy2,

cyanine; FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine.

Figure 16: The synthesis of a photocleavable energy transfer dye-labeled nucleotide. DMF, dimethylformide. DEC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. R = H, CH₂OCH₃ (MOM) or CH₂CH=CH₂ (Allyl).

Figure 17: Structures of four mass tag precursors and four photoactive mass tags. Precursors: a) acetophenone; b) 3-fluoroacetophenone; c) 3,4-difluoroacetophenone; and d) 3,4-dimethoxyacetophenone. Four photoactive mass tags are used to code for the identity of each of the four nucleotides (A, C, G, T).

Figure 18: Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of mass tag precursors shown in Figure 17.

Figure 19: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique mass tag attached to the base through a photocleavable linker, and the 3'-OH is either exposed or capped with a MOM group or an allyl group. The square brackets indicated that the mass tag is cleavable. R = H, CH₂OCH₃ (MOM) or CH₂CH=CH₂ (Allyl).

Figure 20: Example of synthesis of NHS ester of one mass tag (Tag-3). A similar scheme is used to create other mass tags.

Figure 21: A representative scheme for the synthesis of the nucleotide analogue 3'-RO-G-Tag₃. A similar scheme is used to create the other three modified bases 3'-RO-A-Tag₁, 3'-RO-C-Tag₂, 3'-RO-T-Tag₄. (i) tetrakis(triphenylphosphine) palladium(0); (ii) POCl₃, Bn₄N⁺pyrophosphate; (iii) NH₄OH; (iv) Na₂CO₃/NaHCO₃ (pH = 9.0)/DMSO.

Figure 22: Examples of expected photocleavage products of DNA containing a photocleavable mass tag.

Detailed Description Of The Invention

[0017] The following definitions are presented as an aid in understanding this invention.

[0018] As used herein, to **cap** an -OH group means to replace the "H" in the -OH group with a chemical group. As disclosed herein, the -OH group of the nucleotide analogue is capped with a cleavable chemical group. To **uncap** an -OH group means to cleave the chemical group from a capped -OH group and to replace the chemical group with "H", i.e., to replace the "R" in -OR with "H" wherein "R" is the chemical group used to cap the -OH group.

[0019] The nucleotide bases are abbreviated as follows: adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U).

[0020] An **analogue** of a **nucleotide base** refers to a structural and functional derivative of the base of a nucleotide which can be recognized by polymerase as a substrate. That is, for example, an analogue of adenine (A) should form hydrogen bonds with thymine (T), a C analogue should form hydrogen bonds with G, a G analogue should form hydrogen bonds with C, and a T analogue should form hydrogen bonds with A, in a double helix format. Examples of analogues of nucleotide bases include, but are not limited to, 7-deaza-adenine and 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom.

[0021] A **nucleotide analogue** refers to a chemical compound that is structurally and functionally similar to the nucleotide, i.e. the nucleotide analogue can be recognized by polymerase as a substrate. That is, for example, a nucleotide analogue comprising adenine or an analogue of adenine should form hydrogen bonds with thymine, a nucleotide analogue comprising C or an analogue of C should form hydrogen bonds with G, a nucleotide analogue comprising G or an analogue of G should form hydrogen bonds with C, and a nucleotide analogue comprising T or an analogue of T should form hydrogen bonds with A, in a double helix format. Examples of nucleotide analogues disclosed herein include analogues which comprise an analogue of the nucleotide base such as 7-deaza-adenine or 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom. Further examples include analogues in which a label is attached through a cleavable linker to the 5-position of cytosine or thymine or to the 7-position of deaza-adenine or deaza-guanine. Other examples include analogues in which a small chemical moiety such as -CH₂OCH₃ or -CH₂CH=CH₂ is used to cap the -OH group at the 3'-position of deoxyribose. Analogues of dideoxynucleotides can similarly be prepared.

[0022] As used herein, a **porous** surface is a surface which contains pores or is otherwise uneven, such that the surface area of the porous surface is increased relative to the surface area when the surface is smooth.

[0023] The present invention is directed to a method for sequencing a nucleic acid by sequentially determining the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymer-

ase reaction, which comprises the following steps:

- (i) attaching a 5' end of the nucleic acid to a solid surface;
- 5 (ii) attaching a primer to the nucleic acid attached to the solid surface;
- (iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$;
- 10 (iv) washing the solid surface to remove unincorporated nucleotide analogues;
- (v) determining the identity of the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;
- 20 (vi) adding one or more chemical compounds to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;
- (vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;
- 25 (viii) cleaving the cleavable chemical group capping the -OH group at the 3'-position of the deoxyribose to uncapped the -OH group, and washing the solid surface to remove cleaved compounds; and
- 30 (ix) repeating steps (iii) through (viii) so as to determine, for each repetition, the identity of the newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

35 **[0024]** In one embodiment of any of the nucleotide analogues described herein, the nucleotide base is adenine. In one embodiment, the nucleotide base is guanine. In one embodiment, the nucleotide base is cytosine. In one embodiment, the nucleotide base is thymine. In one embodiment, the nucleotide base is uracil. In one embodiment, the nucleotide base is an analogue of adenine. In one embodiment, the nucleotide base is an analogue of guanine. In one embodiment, the nucleotide base is an analogue of cytosine. In one embodiment, the nucleotide base is an analogue of thymine. In one embodiment, the nucleotide base is an analogue of uracil.

40 **[0025]** In different embodiments of any of the inventions described herein, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip. In one embodiment, the solid surface is glass. In one embodiment, the solid surface is silicon. In one embodiment, the solid surface is gold. In one embodiment, the solid surface is a magnetic bead. In one embodiment, the solid surface is a chip. In one embodiment, the solid surface is a channel in a chip. In one embodiment, the solid surface is a porous channel in a chip. Other materials can also be used as long as the material does not interfere with the steps of the method.

45 **[0026]** In one embodiment, the step of attaching the nucleic acid to the solid surface comprises:

- 50 (i) coating the solid surface with a phosphine moiety,
- (ii) attaching an azido group to the 5' end of the nucleic acid, and
- (iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

55 **[0027]** In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

- (i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

[0028] In one embodiment, the nucleic acid that is attached to the solid surface is a single-stranded deoxyribonucleic acid (DNA). In another embodiment, the nucleic acid that is attached to the solid surface in step (i) is a double-stranded DNA, wherein only one strand is directly attached to the solid surface, and wherein the strand that is not directly attached to the solid surface is removed by denaturing before proceeding to step (ii). In one embodiment, the nucleic acid that is attached to the solid surface is a ribonucleic acid (RNA), and the polymerase in step (iii) is reverse transcriptase.

[0029] In one embodiment, the primer is attached to a 3' end of the nucleic acid in step (ii), and the attached primer comprises a stable loop and an -OH group at a 3'-position of a deoxyribose capable of self-priming in the polymerase reaction. In one embodiment, the step of attaching the primer to the nucleic acid comprises hybridizing the primer to the nucleic acid or ligating the primer to the nucleic acid. In one embodiment, the primer is attached to the nucleic acid through a ligation reaction which links the 3' end of the nucleic acid with the 5' end of the primer.

[0030] In one embodiment, one or more of four different nucleotide analogs is added in step (iii), wherein each different nucleotide analogue comprises a different base selected from the group consisting of thymine or uracil or an analogue of thymine or uracil, adenine or an analogue of adenine, cytosine or an analogue of cytosine, and guanine or an analogue of guanine, and wherein each of the four different nucleotide analogues comprises a unique label.

[0031] The cleavable chemical group that caps the -OH group at the 3'-position of the deoxyribose in the nucleotide analogue is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$. Any one of these chemical groups 1) is stable during the polymerase reaction, 2) does not interfere with the recognition of the nucleotide analogue by polymerase as a substrate, and 3) is cleavable.

[0032] In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine. In one embodiment, the fluorescent moiety is 5-carboxyfluorescein. In one embodiment, the fluorescent moiety is 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the fluorescent moiety is 6-carboxy-X-rhodamine.

[0033] In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarbonyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine. In one embodiment, the energy transfer acceptor is dichlorocarbonyfluorescein. In one embodiment, the energy transfer acceptor is dichloro-6-carboxyrhodamine-6G. In one embodiment, the energy transfer acceptor is dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the energy transfer acceptor is dichloro-6-carboxy-X-rhodamine.

[0034] In one embodiment, the unique label that is attached to the nucleotide analogue is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group consisting of a 2-nitro- α -methyl-benzyl group, a 2-nitro- α -methyl-3-fluorobenzyl group, a 2-nitro- α -methyl-3,4-difluorobenzyl group, and a 2-nitro- α -methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is a 2-nitro- α -methyl-benzyl group. In one embodiment, the mass tag is a 2-nitro- α -methyl-3-fluorobenzyl group. In one embodiment, the mass tag is a 2-nitro- α -methyl-3,4-difluorobenzyl group. In one embodiment, the mass tag is a 2-nitro- α -methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is detected using a parallel mass spectrometry system which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.

[0035] In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

[0036] In one embodiment, the linker between the unique label and the nucleotide analogue is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical means. In one embodiment, the linker is cleaved by a chemical means. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

[0037] In one embodiment, the cleavable chemical group used to cap the -OH group at the 3'-position of the deoxyribose is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment,

the linker is cleaved by ultraviolet light.

[0038] In one embodiment, the chemical compounds added in step (vi) to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on the primer extension strand are a polymerase and one or more different dideoxynucleotides or analogues of dideoxynucleotides. In further embodiments, the different dideoxynucleotides are selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine 5'-triphosphate, and their analogues. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphate.

[0039] In one embodiment, a polymerase and one or more of four different dideoxynucleotides are added in step (vi), wherein each different dideoxynucleotide is selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate or an analogue of 2',3'-dideoxyadenosine 5'-triphosphate; 2',3'-dideoxyguanosine 5'-triphosphate or an analogue of 2',3'-dideoxyguanosine 5'-triphosphate; 2',3'-dideoxycytidine 5'-triphosphate or an analogue of 2',3'-dideoxycytidine 5'-triphosphate; and 2',3'-dideoxythymidine 5'-triphosphate or 2',3'-dideoxyuridine 5'-triphosphate or an analogue of 2',3'-dideoxythymidine 5'-triphosphate or an analogue of 2',3'-dideoxyuridine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphate.

[0040] Another type of chemical compound that reacts specifically with the -OH group could also be used to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on an extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer.

[0041] The invention provides a method for simultaneously sequencing a plurality of different nucleic acids, which comprises simultaneously applying any of the methods disclosed herein for sequencing a nucleic acid to the plurality of different nucleic acids. In different embodiments, the method can be used to sequence from one to over 100,000 different nucleic acids simultaneously.

[0042] The invention provides for the use of any of the methods disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

[0043] The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

- (i) coating the solid surface with a phosphine moiety,
- (ii) attaching an azido group to a 5' end of the nucleic acid, and
- (iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

[0044] In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

- (i) coating the surface with a primary amine, and
- (ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

[0045] In different embodiments, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip.

[0046] In different embodiments, the nucleic acid that is attached to the solid surface is a single-stranded or double-stranded DNA or a RNA. In one embodiment, the nucleic acid is a double-stranded DNA and only one strand is attached

to the solid surface. In a further embodiment, the strand of the double-stranded DNA that is not attached to the solid surface is removed by denaturing.

[0047] The invention provides for the use of any of the methods disclosed herein for attaching a nucleic acid to a surface for gene expression analysis, microarray based gene expression analysis, or mutation detection, translational analysis, transcriptional analysis, or for other genetic applications.

[0048] The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

[0049] In one embodiment of the nucleotide analogue, the cleavable chemical group that caps the -OH group at the 3'-position of the deoxyribose is $-\text{CH}_2\text{CH}=\text{CH}_2$.

[0050] In one embodiment, the unique label is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine.

[0051] In one embodiment, the unique label is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine.

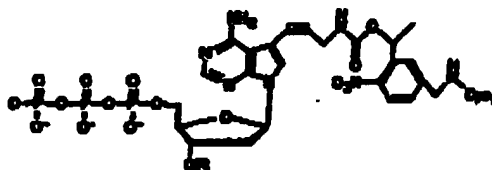
[0052] In one embodiment, the unique label is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group consisting of a 2-nitro- α -methyl-benzyl group, a 2-nitro- α -methyl-3-fluorobenzyl group, a 2-nitro- α -methyl-3,4-difluorobenzyl group, and a 2-nitro- α -methyl-3,4-dimethoxybenzyl group.

[0053] In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

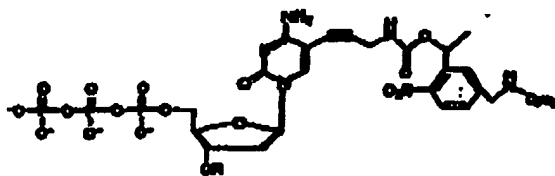
[0054] In one embodiment, the linker between the unique label and the nucleotide analogue is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

[0055] In one embodiment, the cleavable chemical group used to cap the -OH group at the 3'-position of the deoxyribose is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.

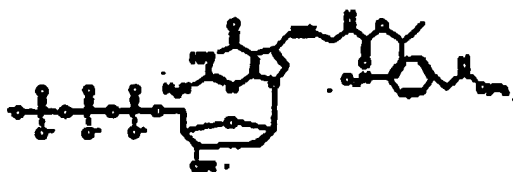
[0056] In different embodiments, the nucleotide analogue is selected from the group consisting of:



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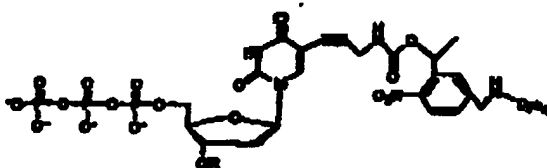
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and

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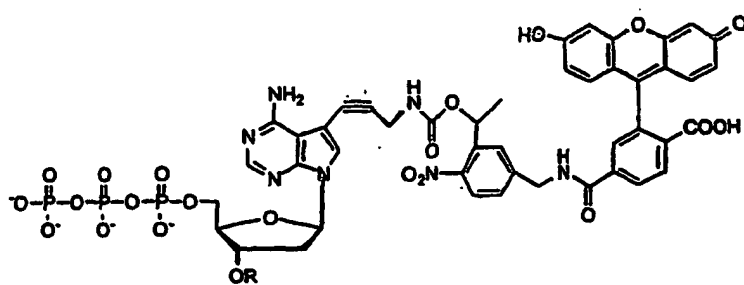
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wherein Dye₁, Dye₂, Dye₃, and Dye₄ are four different unique labels; and
wherein R is -CH₂OCH₃ or -CH₂CH=CH₂.

[0057] In different embodiments, the nucleotide analogue is selected from the group consisting of:

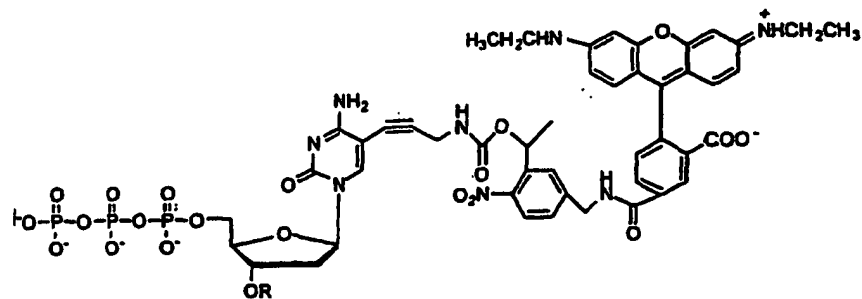
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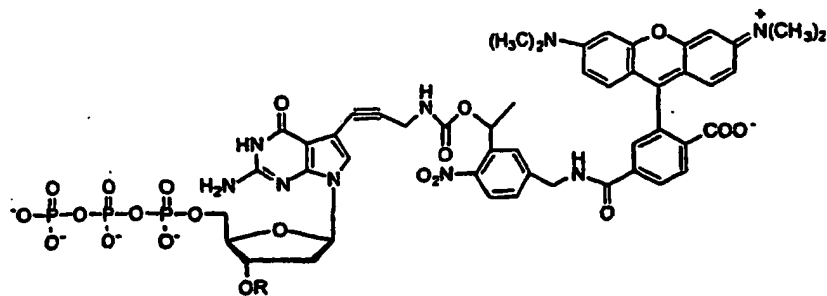
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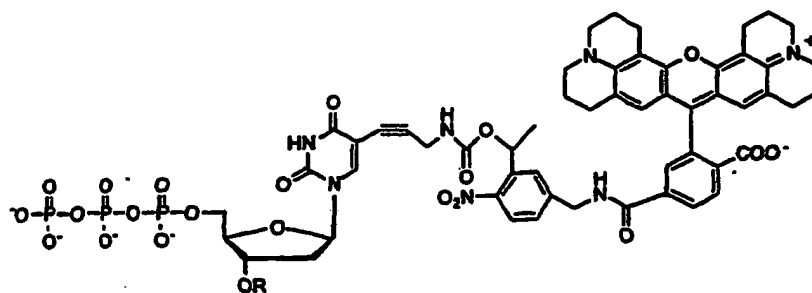
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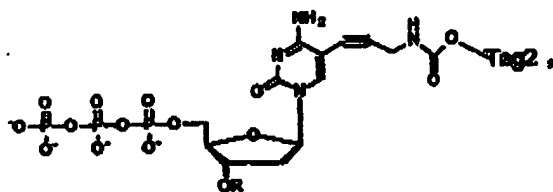
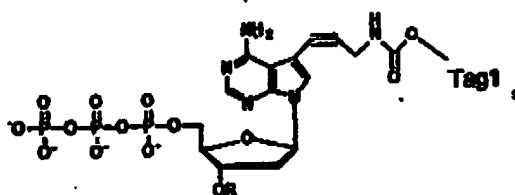


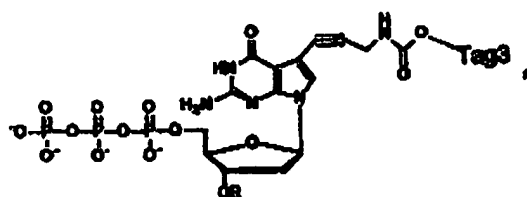
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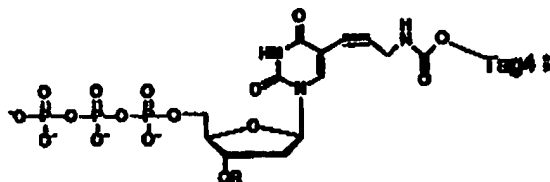
wherein R is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

[0058] In different embodiments, the nucleotide analogue is selected from the group consisting of:



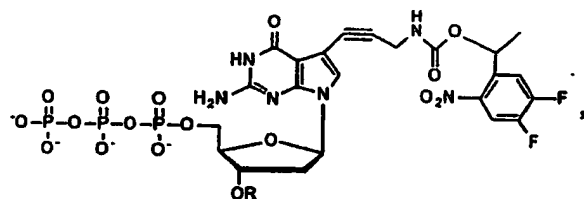
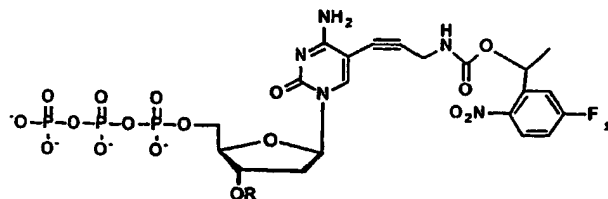
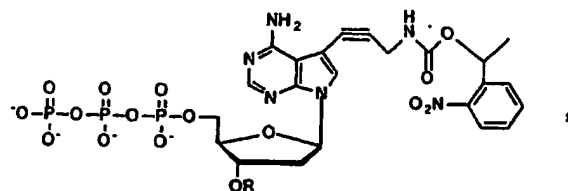


and

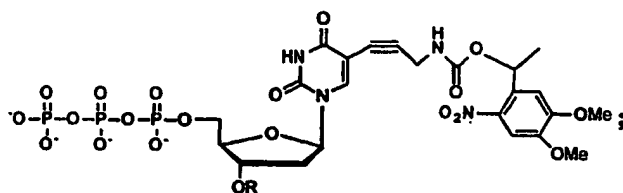


wherein Tag₁, Tag₂, Tag₃, and Tag₄ are four different mass tag labels; and
 wherein R is -CH₂OCH₃ or -CH₂CH=CH₂.

[0059] In different embodiments, the nucleotide analogue is selected from the group consisting of:



and



wherein R is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

[0060] The invention provides for the use any of the nucleotide analogues disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

Experimental Details

1. The Sequencing by Synthesis Approach

[0061] Sequencing DNA by synthesis involves the detection of the identity of each nucleotide as it is incorporated into the growing strand of DNA in the polymerase reaction. The fundamental requirements for such a system to work are: (1) the availability of 4 nucleotide analogues (aA, aC, aG, aT) each labeled with a unique label and containing a chemical moiety capping the 3'-OH group; (2) the 4 nucleotide analogues (aA, aC, aG, aT) need to be efficiently and faithfully incorporated by DNA polymerase as terminators in the polymerase reaction; (3) the tag and the group capping the 3'-OH need to be removed with high yield to allow the incorporation and detection of the next nucleotide; and (4) the growing strand of DNA should survive the washing, detection and cleavage processes to remain annealed to the DNA template.

[0062] The sequencing by synthesis approach disclosed herein is illustrated in **Figure 2A-2B**. In **Figure 2A**, an example is shown where the unique labels are fluorescent dyes and the surface is a chip; in **Figure 2B**, the unique labels are mass tags and the surface is channels etched into a chip. The synthesis approach uses a solid surface such as a glass chip with an immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues ($3'\text{-RO-A-LABEL1}$, $3'\text{-RO-C-LABEL2}$, $3'\text{-RO-G-LABEL3}$, $3'\text{-RO-T-LABEL4}$) each labeled with a unique label, e.g. a fluorescent dye or a mass tag, at a specific location on the purine or pyrimidine base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by the polymerase on each spot of the surface (**step 1 in Fig. 2A and 2B**).

[0063] As shown in **Figure 2A**, where the unique labels are dyes, after removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, a detector is used to detect the unique label. For example, a four color fluorescence imager is used to image the surface of the chip, and the unique fluorescence emission from a specific dye on the nucleotide analogues on each spot of the chip will reveal the identity of the incorporated nucleotide (**step 2 in Fig. 2A**). After imaging, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess dideoxynucleoside triphosphates (ddNTPs) (ddATP, ddGTP, ddTTP, and ddCTP) and DNA polymerase to avoid interference with the next round of synthesis (**step 3 in Fig. 2A**), a concept similar to the capping step in automated solid phase DNA synthesis (Caruthers, 1985). The ddNTPs, which lack a 3'-hydroxyl group, are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the dye-labeled nucleotides, and the excellent efficiency with which they are incorporated by DNA polymerase. The dye moiety is then cleaved by light (~350 nm), and the R group protecting the 3'-OH is removed chemically to generate free 3'-OH group with high yield (**step 4 in Fig. 2A**). A washing step is applied to wash away the cleaved dyes and the R group. The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (**step 5 in Fig. 2A**).

[0064] It is a routine procedure now to immobilize high density (>10,000 spots per chip) single stranded DNA on a 4cm x 1cm glass chip (Schna et al. 1995). Thus, in the DNA sequencing system disclosed herein, more than 10,000 bases can be identified after each cycle and after 100 cycles, a million base pairs will be generated from one sequencing chip.

[0065] Possible DNA polymerases include Thermo Sequenase, Taq FS DNA polymerase, T7 DNA polymerase, and Vent (exo-) DNA polymerase. The fluorescence emission from each specific dye can be detected using a fluorimeter that is equipped with an accessory to detect fluorescence from a glass slide. For large scale evaluation, a multi-color scanning system capable of detecting multiple different fluorescent dyes (500 nm - 700 nm) (GSI Lumonics ScanArray

5000 Standard Biochip Scanning System) on a glass slide can be used.

[0066] An example of the sequencing by synthesis approach using mass tags is shown in **Figure 2B**. The approach uses a solid surface, such as a porous silica glass channels in a chip, with immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues (3'-RO-A-Tag1, 3'-RO-C-Tag2, 3'-RO-G-Tag3, 3'-RO-T-Tag4) each labeled with a unique photocleavable mass tag on the specific location of the base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by polymerase in each channel of the glass chip (**step 1 in Fig. 2B**). After removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess ddNTPs (ddATP, ddGTP, ddTTP and ddCTP) and DNA polymerase to avoid interference with the next round of synthesis (**step 2 in Fig. 2B**). The ddNTPs are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the labeled nucleotides, and their excellent efficiency to be incorporated by DNA polymerase. The mass tags are cleaved by irradiation with light (~350 nm) (**step 3 in Fig. 2B**) and then detected with a mass spectrometer. The unique mass of each tag yields the identity of the nucleotide in each channel (**step 4 in Fig. 2B**). The R protecting group is then removed chemically and washed away to generate free 3'-OH group with high yield (**step 5 in Fig. 2B**). The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (**step 6 in Fig. 2B**).

[0067] Since the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), mass spectrometry has become an indispensable tool in many areas of biomedical research. Though these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation are required for implementation of mass spectrometry for DNA sequencing applications. Since the approach disclosed herein uses small and stable mass tags, there is no need to detect large DNA sequencing fragments directly and it is not necessary to use MALDI or ESI methods for detection. Atmospheric pressure chemical ionization (APCI) is an ionization method that uses a gas-phase ion-molecular reaction at atmospheric pressure (Dizidic et al. 1975). In this method, samples are introduced by either chromatography or flow injection into a pneumatic nebulizer where they are converted into small droplets by a high-speed beam of nitrogen gas. When the heated gas and solution arrive at the reaction area, the excess amount of solvent is ionized by corona discharge. This ionized mobile phase acts as the ionizing agent toward the samples and yields pseudo molecular (M+H)⁺ and (M-H)⁻ ions. Due to the corona discharge ionization method, high ionization efficiency is attainable, maintaining stable ionization conditions with detection sensitivity lower than femtomole region for small and stable organic compounds. However, due to the limited detection of large molecules, ESI and MALDI have replaced APCI for analysis of peptides and nucleic acids. Since in the approach disclosed the mass tags to be detected are relatively small and very stable organic molecules, the ability to detect large biological molecules gained by using ESI and MALDI is not necessary. APCI has several advantages over ESI and MALDI because it does not require any tedious sample preparation such as desalting or mixing with matrix to prepare crystals on a target plate. In ESI, the sample nature and sample preparation conditions (i.e. the existence of buffer or inorganic salts) suppress the ionization efficiency. MALDI requires the addition of matrix prior to sample introduction into the mass spectrometer and its speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. These limitations are overcome by APCI because the mass tag solution can be injected directly with no additional sample purification or preparation into the mass spectrometer. Since the mass tagged samples are volatile and have small mass numbers, these compounds are easily detectable by APCI ionization with high sensitivity. This system can be scaled up into a high throughput operation.

[0068] Each component of the sequencing by synthesis system is described in more detail below.

2. Construction of a Surface Containing Immobilized Self-primed DNA Moiety

[0069] The single stranded DNA template immobilized on a surface is prepared according to the scheme shown in **Figure 3**. The surface can be, for example, a glass chip, such as a 4cm x 1cm glass chip, or channels in a glass chip. The surface is first treated with 0.5 M NaOH, washed with water, and then coated with high density 3-aminopropyltrimethoxysilane in aqueous ethanol (Woolley et al. 1994) forming a primary amine surface. N-Hydroxy Succinimidyl (NHS) ester of triarylphosphine (1) is covalently coupled with the primary amine group converting the amine surface to a novel triarylphosphine surface, which specifically reacts with DNA containing an azido group (2) forming a chip with immobilized DNA. Since the azido group is only located at the 5' end of the DNA and the coupling reaction is through the unique reaction of the triarylphosphine moiety with the azido group in aqueous solution (Saxon and Bertozzi 2000), such a DNA surface will provide an optimal condition for hybridization.

[0070] The NHS ester of triarylphosphine (1) is prepared according to the scheme shown in **Figure 4**. 3-diphenylphosphino-4-methoxycarbonyl-benzoic acid (3) is prepared according to the procedure described by Bertozzi et al. (Saxon and Bertozzi 2000). Treatment of (3) with N-Hydroxysuccinimide forms the corresponding NHS ester (4). Coupling

of (4) with an amino carboxylic acid moiety produces compound (5) that has a long linker ($n = 1$ to 10) for optimized coupling with DNA on the surface. Treatment of (5) with N-Hydroxysuccinimide generates the NHS ester (1) which is ready for coupling with the primary amine coated surface (Figure 3).

[0071] The azido labeled DNA (2) is synthesized according to the scheme shown in Figure 5. Treatment of ethyl ester of 5-bromovaleric acid with sodium azide and then hydrolysis produces 5-azidovaleric acid (Khoukhi et al., 1987), which is subsequently converted to a NHS ester for coupling with an amino linker modified oligonucleotide primer. Using the azido-labeled primer to perform polymerase chain reaction (PCR) reaction generates azido-labeled DNA template (2) for coupling with the triarylphosphine-modified surface (Figure 3).

[0072] The self-primed DNA template moiety on the sequencing chip is constructed as shown in Figure 6 (A & B) using enzymatic ligation. A 5'-phosphorylated, 3'-OH capped loop oligonucleotide primer (B) is synthesized by a solid phase DNA synthesizer. Primer (B) is synthesized using a modified C phosphoramidite whose 3'-OH is capped with either a MOM ($-\text{CH}_2\text{OCH}_3$) group or an allyl ($-\text{CH}_2\text{CH}=\text{CH}_2$) group (designated by "R" in Figure 6) at the 3'-end of the oligonucleotide to prevent the self ligation of the primer in the ligation reaction. Thus, the looped primer can only ligate to the 3'-end of the DNA templates that are immobilized on the sequencing chip using T4 RNA ligase (Zhang et al. 1996) to form the self-primed DNA template moiety (A). The looped primer (B) is designed to contain a very stable loop (Antao et al. 1991) and a stem containing the sequence of M13 reverse DNA sequencing primer for efficient priming in the polymerase reaction once the primer is ligated to the immobilized DNA on the sequencing chip and the 3'-OH cap group is chemically cleaved off (Ireland et al. 1986; Kamal et al. 1999).

3. Sequencing by Synthesis Evaluation Using Nucleotide

Analogues $3'\text{-HO-A-Dye1}$, $3'\text{-HO-C-Dye2}$, $3'\text{-HO-G-Dye3}$, $3'\text{-HO-T-Dye4}$

[0073] A scheme has been developed for evaluating the photocleavage efficiency using different dyes and testing the sequencing by synthesis approach. Four nucleotide analogues $3'\text{-HO-A-Dye1}$, $3'\text{-HO-C-Dye2}$, $3'\text{-HO-G-Dye3}$, $3'\text{-HO-T-Dye4}$ each labeled with a unique fluorescent dye through a photocleavable linker are synthesized and used in the sequencing by synthesis approach. Examples of dyes include, but are not limited to: Dye1 = FAM, 5-carboxyfluorescein; Dye2 = R6G, 6-carboxyrhodamine-6G; Dye3 = TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; and Dye4 = ROX, 6-carboxy-X-rhodamine. The structures of the 4 nucleotide analogues are shown in Figure 7 ($R = h$).

[0074] The photocleavable 2-nitrobenzyl moiety has been used to link biotin to DNA and protein for efficient removal by UV light (~ 350 nm) (Olejnik et al. 1995, 1999). In the approach disclosed herein the 2-nitrobenzyl group is used to bridge the fluorescent dye and nucleotide together to form the dye labeled nucleotides as shown in Figure 7.

[0075] As a representative example, the synthesis of $3'\text{-HO-G-Dye3}$ (Dye3 = Tam) is shown in Figure 8. 7-deaza-alkynylamino-dGTP is prepared using well-established procedures (Prober et al. 1987; Lee et al. 1992 and Hobbs et al. 1991). Linker-Tam is synthesized by coupling the Photocleavable Linker (Rollaf 1982) with NHS-Tam. 7-deaza-alkynylamino-dGTP is then coupled with the Linker-Tam to produce $3'\text{-HO-G-TAM}$. The nucleotide analogues with a free 3'-OH (i.e., $R = H$) are good substrates for the polymerase. An immobilized DNA template is synthesized (Figure 9) that contains a portion of nucleotide sequence ACGTACGACGT (SEQ ID NO: 1) that has no repeated sequences after the priming site. $3'\text{-HO-A-Dye1}$ and DNA polymerase are added to the self-primed DNA moiety and it is incorporated to the 3' site of the DNA. Then the steps in Figure 2A are followed (the chemical cleavage step is not required here because the 3'-OH is free) to detect the fluorescent signal from Dye-1 at 520 nm. Next, $3'\text{-HO-C-Dye2}$ is added to image the fluorescent signal from Dye-2 at 550 nm. Next, $3'\text{-HO-G-Dye3}$ is added to image the fluorescent signal from Dye-3 at 580 nm, and finally $3'\text{-HO-T-Dye4}$ is added to image the fluorescent signal from Dye-4 at 610 nm.

Results on photochemical cleavage efficiency

[0076] The expected photolysis products of DNA containing a photocleavable fluorescent dye at the 3' end of the DNA are shown in Figure 10. The 2-nitrobenzyl moiety has been successfully employed in a wide range of studies as a photocleavable-protecting group (Pillai 1980). The efficiency of the photocleavage step depends on several factors including the efficiency of light absorption by the 2-nitrobenzyl moiety, the efficiency of the primary photochemical step, and the efficiency of the secondary thermal processes which lead to the final cleavage process (Turro 1991). Burgess et al. (1997) have reported the successful photocleavage of a fluorescent dye attached through a 2-nitrobenzyl linker on a nucleotide moiety, which shows that the fluorescent dye is not quenching the photocleavage process. A photolabile protecting group based on the 2-nitrobenzyl chromophore has also been developed for biological labeling applications that involve photocleavage (Olejnik et al. 1999). The protocol disclosed herein is used to optimize the photocleavage process shown in Figure 10. The absorption spectra of 2-nitro benzyl compounds are examined and compared quantitatively to the absorption spectra of the fluorescent dyes. Since there will be a one-to-one relationship between the number of 2-nitrobenzyl moieties and the dye molecules, the ratio of extinction coefficients of these two species will reflect the competition for light absorption at specific wavelengths. From this information, the wavelengths at which the

2-nitrobenzyl moieties absorbed most competitively can be determined, similar to the approach reported by Olejnik et al. (1995).

[0077] A photolysis setup can be used which allows a high throughput of monochromatic light from a 1000 watt high pressure xenon lamp (LX1000UV, ILC) in conjunction with a monochromator (Kratos, Schoeffel Instruments). This instrument allows the evaluation of the photocleavage of model systems as a function of the intensity and excitation wavelength of the absorbed light. Standard analytical analysis is used to determine the extent of photocleavage. From this information, the efficiency of the photocleavage as a function of wavelength can be determined. The wavelength at which photocleavage occurs most efficiently can be selected as for use in the sequencing system.

[0078] Photocleavage results have been obtained using a model system as shown in Figure 11. Coupling of **PC-LC-Biotin-NHS** ester (Pierce, Rockford IL) with 5-(aminoacetamido)-fluorescein (5-aminoFAM) (Molecular Probes, Eugene OR) in dimethylsulfonyl oxide (DMSO)/NaHCO₃ (pH=8.2) overnight at room temperature produces **PC-LC-Biotin-FAM** which is composed of a biotin at one end, a photocleavable 2-nitrobenzyl group in the middle, and a dye tag (FAM) at the other end. This photocleavable moiety closely mimics the designed photocleavable nucleotide analogues shown in Figure 10. Thus the successful photolysis of the **PC-LC-Biotin-FAM** moiety provides proof of the principle of high efficiency photolysis as used in the DNA sequencing system. For photolysis study, **PC-LC-Biotin-FAM** is first immobilized on a microscope glass slide coated with streptavidin (XENOPORE, Hawthorne NJ). After washing off the non-immobilized **PC-LC-Biotin-FAM**, the fluorescence emission spectrum of the immobilized **PC-LC-Biotin-FAM** was taken as shown in Figure 12 (Spectrum a). The strong fluorescence emission indicates that **PC-LC-Biotin-FAM** is successfully immobilized to the streptavidin coated slide surface. The photocleavability of the 2-nitrobenzyl linker by irradiation at 350 nm was then tested. After 10 minutes of photolysis ($\lambda_{irr} = 350 \text{ nm}$; $\sim 0.5 \text{ mW/cm}^2$) and before any washing, the fluorescence emission spectrum of the same spot on the slide was taken that showed no decrease in intensity (Figure 12, Spectrum b), indicating that the dye (FAM) was not bleached during the photolysis process at 350 nm. After washing the glass slide with HPLC water following photolysis, the fluorescence emission spectrum of the same spot on the slide showed significant intensity decrease (Figure 12, Spectrum c) which indicates that most of the fluorescence dye (FAM) was cleaved from the immobilized biotin moiety and was removed by the washing procedure. This experiment shows that high efficiency cleavage of the fluorescent dye can be obtained using the 2-nitrobenzyl photocleavable linker.

4. Sequencing by Synthesis Evaluation Using Nucleotide

Analogues 3'-RO-A-Dye1, 3'-RO-C-Dye2, 3'-RO-G-Dye3, 3'-RO-T-Dye4

[0079] Once the steps and conditions in Section 3 are optimized, the synthesis of nucleotide analogues 3'-RO-A-Dye1, 3'-RO-C-Dye2, 3'-RO-G-Dye3, 3'-RO-T-Dye4 can be pursued for further study of the system. Here the 3'-OH is capped in all four nucleotide analogues, which then can be mixed together with DNA polymerase and used to evaluate the sequencing system using the scheme in Figure 9. The MOM (-CH₂OCH₃) or allyl (-CH₂CH=CH₂) group is used to cap the 3'-OH group using well-established synthetic procedures (Figure 13) (Fuji et al. 1975, Metzker et al. 1994). These groups can be removed chemically with high yield as shown in Figure 14 (Ireland, et al. 1986; Kamal et al. 1999). The chemical cleavage of the MOM and allyl groups is fairly mild and specific, so as not to degrade the DNA template moiety. For example, the cleavage of the allyl group takes 3 minutes with more than 93% yield (Kamal et al. 1999), while the MOM group is reported to be cleaved with close to 100% yield (Ireland, et al. 1986).

5. Using Energy Transfer Coupled Dyes To Optimize The Sequencing By Synthesis System

[0080] The spectral property of the fluorescent tags can be optimized by using energy transfer (ET) coupled dyes.

[0081] The ET primer and ET dideoxynucleotides have been shown to be a superior set of reagents for 4-color DNA sequencing that allows the use of one laser to excite multiple sets of fluorescent tags (Ju et al. 1995). It has been shown that DNA polymerase (Thermo Sequenase and Taq FS) can efficiently incorporate the ET dye labeled dideoxynucleotides (Rosenblum et al. 1997). These ET dye-labeled sequencing reagents are now widely used in large scale DNA sequencing projects, such as the human genome project. A library of ET dye labeled nucleotide analogues can be synthesized as shown in Figure 15 for optimization of the DNA sequencing system. The ET dye set (FAM-Cl₂FAM, FAM-Cl₂R6G, FAM-Cl₂TAM, FAM-Cl₂ROX) using FAM as a donor and dichloro(FAM, R6G, TAM, ROX) as acceptors has been reported in the literature (Lee et al. 1997) and constitutes a set of commercially available DNA sequencing reagents. These ET dye sets have been proven to produce enhanced fluorescence intensity, and the nucleotides labeled with these ET dyes at the 5-position of T and C and the 7-position of G and A are excellent substrates of DNA polymerase. Alternatively, an ET dye set can be constructed using cyanine (Cy2) as a donor and Cl₂FAM, Cl₂R6G, Cl₂TAM, or Cl₂ROX as energy acceptors. Since Cy2 possesses higher molar absorbance compared with the rhodamine and fluorescein derivatives, an ET system using Cy2 as a donor produces much stronger fluorescence signals than the system using FAM as a donor (Hung et al. 1996). Figure 16 shows a synthetic scheme for an ET dye labeled nucleotide analogue with Cy2 as a donor and Cl₂FAM as an acceptor using similar coupling chemistry as for the synthesis of an energy transfer system

using FAM as a donor (Lee et al. 1997). Coupling of Cl₂FAM (I) with spacer 4-aminomethylbenzoic acid (II) produces III, which is then converted to NHS ester IV. Coupling of IV with amino-Cy2, and then converting the resulting compound to a NHS ester produces V, which subsequently couples with amino-photolinker nucleotide VI yields the ET dye labeled nucleotide VII.

6. Sequencing by synthesis evaluation using nucleotide

analogues 3'-HO-A-Tag₁, 3'-HO-C-Tag₂, 3'-HO-G-Tag₃, 3'-HO-T-Tag₄

[0082] The precursors of four examples of mass tags are shown in Figure 17. The precursors are: (a) acetophenone; (b) 3-fluoroacetophenone; (c) 3,4-difluoroacetophenone; and (d) 3,4-dimethoxyacetophenone. Upon nitration and reduction, four photoactive tags are produced from the four precursors and used to code for the identity of each of the four nucleotides (A, C, G, T). Clean APCI mass spectra are obtained for the four mass tag precursors (a, b, c, d) as shown in Figure 18. The peak with m/z of 121 is a, 139 is b, 157 is c, and 181 is d. This result shows that these four mass tags are extremely stable and produce very high resolution data in an APCI mass spectrometer with no cross talk between the mass tags. In the examples shown below, each of the unique m/z from each mass tag translates to the identity of the nucleotide [Tag-1 (m/z, 150) = A; Tag-2 (m/z, 168) = C; Tag-3 (m/z, 186) = G; Tag-4 (m/z, 210) = T].

[0083] Different combinations of mass tags and nucleotides can be used, as indicated by the general scheme: 3'-HO-A-Tag₁, 3'-HO-C-Tag₂, 3'-HO-G-Tag₃, 3'-HO-T-Tag₄ where Tag₁, Tag₂, Tag₃, and Tag₄ are four different unique cleavable mass tags. Four specific examples of nucleotide analogues are shown in Figure 19. In Figure 19, "R" is H when the 3'-OH group is not capped. As discussed above, the photo cleavable 2-nitro benzyl moiety has been used to link biotin to DNA and protein for efficient removal by UV light (~ 350 nm) irradiation (Olejnik et al. 1995, 1999). Four different 2-nitro benzyl groups with different molecular weights as mass tags are used to form the mass tag labeled nucleotides as shown in Figure 19: 2-nitro- α -methyl-benzyl (Tag-1) codes for A; 2-nitro- α -methyl-3-fluorobenzyl (Tag-2) codes for C; 2-nitro- α -methyl-3,4-difluorobenzyl (Tag-3) codes for G; 2-nitro- α -methyl-3,4-dimethoxybenzyl (Tag-4) codes for T.

[0084] As a representative example, the synthesis of the NHS ester of one mass tag (Tag-3) is shown in Figure 20. A similar scheme is used to create the other mass tags. The synthesis of 3'-HO-G-Tag₃ is shown in Figure 21 using well-established procedures (Prober et al. 1987; Lee et al. 1992 and Hobbs et al. 1991). 7-propargylamino- dGTP is first prepared by reacting 7-I-dGTP with N-trifluoroacetylpropargyl amine, which is then coupled with the NHS-Tag-3 to produce 3'-HO-G-Tag₃. The nucleotide analogues with a free 3'-OH are good substrates for the polymerase.

[0085] The sequencing by synthesis approach can be tested using mass tags using a scheme similar to that shown for dyes in Figure 9. A DNA template containing a portion of nucleotide sequence that has no repeated sequences after the priming site, is synthesized and immobilized to a glass channel. 3'-HO-A-Tag₁ and DNA polymerase are added to the self-primed DNA moiety to allow the incorporation of the nucleotide into the 3' site of the DNA. Then the steps in Figure 2B are followed (the chemical cleavage is not required here because the 3'-OH is free) to detect the mass tag from Tag-1 (m/z = 150). Next, 3'-HO-C-Tag₂ is added and the resulting mass spectra is measured after cleaving Tag-2 (m/z = 168). Next, 3'-HO-G-Tag₃ and 3'-HO-T-Tag₄ are added in turn and the mass spectra of the cleavage products Tag-3 (m/z = 186) and Tag-4 (m/z = 210) are measured. Examples of expected photocleavage products are shown in Figure 22. The photocleavage mechanism is as described above for the case where the unique labels are dyes. Light absorption (300 - 360 nm) by the aromatic 2-nitro benzyl moiety causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond located in the 2-position followed by cleavage and decarboxylation (Pillai 1980).

[0086] The synthesis of nucleotide analogues 3'-RO-A-Tag₁, 3'-RO-C-Tag₂, 3'-RO-G-Tag₃, 3'-RO-T-Tag₄ can be pursued for further study of the system as discussed above for the case where the unique labels are dyes. Here the 3'-OH is capped in all four nucleotide analogues, which then can be mixed together with DNA polymerase and used to evaluate the sequencing system using a scheme similar to that in Figure 9. The MOM (-CH₂OCH₃) or allyl (-CH₂CH=CH₂) group is used to cap the 3'-OH group using well-established synthetic procedures (Figure 13) (Fuji et al. 1975, Metzker et al. 1994). These groups can be removed chemically with high yield as shown in Figure 14 (Ireland, et al. 1986; Kamal et al. 1999). The chemical cleavage of the MOM and allyl groups is fairly mild and specific, so as not to degrade the DNA template moiety.

8. Validate the Complete Sequencing by Synthesis System By Sequencing P53 Genes

[0087] The tumor suppressor gene p53 can be used as a model system to validate the DNA sequencing system. The p53 gene is one of the most frequently mutated genes in human cancer (O'Connor et al. 1997). First, a base pair DNA template (shown below) is synthesized containing an azido group at the 5' end and a portion of the sequences from exon 7 and exon 8 of the p53 gene:

5'-N₃-TTCCTGCATGGGCGGCATGAACCCGAGGCCCATCCTCACCATCATCAC

ACTGGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCATT -3' (SEQ ID NO: 2).

[0088] This template is chosen to explore the use of the sequencing system for the detection of clustered hot spot single base mutations. The potentially mutated bases are underlined (A, G, C and T) in the synthetic template. The synthetic template is immobilized on a sequencing chip or glass channels, then the loop primer is ligated to the immobilized template as described in **Figure 6**, and then the steps in **Figure 2** are followed for sequencing evaluation. DNA templates generated by PCR can be used to further validate the DNA sequencing system. The sequencing templates can be generated by PCR using flanking primers (one of the pair is labeled with an azido group at the 5' end) in the intron region located at each p53 exon boundary from a pool of genomic DNA (Boehringer, Indianapolis, IN) as described by Fu et al. (1998) and then immobilized on the DNA chip for sequencing evaluation.

References

[0089]

- Antao VP, Lai SY, Tinoco I Jr. (1991) A thermodynamic study of unusually stable RNA and DNA hairpins. *Nucleic Acids Res.* **19**: 5901-5905.
- Axelrod VD, Vartikyan RM, Aivazashvili VA, Beabealashvili RS. (1978) Specific termination of RNA polymerase synthesis as a method of RNA and DNA sequencing. *Nucleic Acids Res.* **5**(10): 3549-3563.
- Badman ER and Cooks RG. (2000) Cylindrical Ion Trap Array with Mass Selection by Variation in Trap Dimensions *Anal. Chem.* **72**(20):5079-5086.
- Badman ER and Cooks RG. (2000) A Parallel Miniature Cylindrical Ion Trap Array. *Anal. Chem.* **72**(14):3291-3297.
- Bowling JM, Bruner KL, Cmarik JL, Tibbetts C. (1991) Neighboring nucleotide interactions during DNA sequencing gel electrophoresis. *Nucleic Acids Res.* **19**: 3089-3097.
- Burgess K, Jacutin SE, Lim D, Shitangkoon A. (1997) An approach to photolabile, fluorescent protecting groups. *J. Org. Chem.* **62**(15): 5165-5168.
- Canard B, Cardona B, Sarfati RS. (1995) Catalytic editing properties of DNA polymerases. *Proc. Natl. Acad. Sci. USA* **92**: 10859-10863.
- Caruthers MH. (1985) Gene synthesis machines: DNA chemistry and its uses. *Science* **230**: 281-285.
- Chee M, Yang R, Hubbell E, Berno, A, Huang, XC., Stern D, Winkler, J, Lockhart DJ, Morris M S, Fodor, SP. (1996) Accessing genetic information with high-density DNA arrays. *Science.* **274**: 610-614.
- Cheeseman PC. Method For Sequencing Polynucleotides, United States Patent No. 5,302,509, issued April 12, 1994.
- Dizdovic I, Carrol, DI, Stillwell, RN, and Horning, MG. (1975) Atmospheric pressure ionization (API) mass spectrometry: formation of phenoxide ions from chlorinated aromatic compounds *Anal. Chem.*, **47**:1308-1312.
- Fu DJ, Tang K, Braun A, Reuter D, Darnhofer-Demar B, Little DP, O'Donnell MJ, Cantor CR, Koster H. (1998) Sequencing exons 5 to 8 of the p53 gene by MALDI-TOF mass spectrometry. *Nat Biotechnol.* **16**: 381-384.
- Fuji K, Nakano S, Fujita E. (1975) An improved method for methoxymethylation of alcohols under mild acidic conditions. *Synthesis* 276-277.
- Hobbs FW Jr, Cocuzza AJ. Alkynylamino-Nucleotides. United States Patent No. 5,047,519, issued September 10, 1991.
- Hung SC; Ju J; Mathies RA; Glazer AN. (1996) Cyanine dyes with high absorption cross section as donor chromophores in energy transfer primers. *Anal Biochem.* **243**(1) : 15-27.
- Hyman ED, (1988) A new method of sequencing DNA. *Analytical Biochemistry* **174**: 423-436.

- Ireland RE, Varney MD (1986) Approach to the total synthesis of chlorothricolide-synthesis of (+/-)-19.20-dihydro-24-O-methylchlorothricolide, methyl-ester, ethyl carbonate. *J. Org. Chem.* **51**: 635-648.
- 5 Ju J, Glazer AN, Mathies RA. (1996) Cassette labeling for facile construction of energy transfer fluorescent primers. *Nucleic Acids Res.* **24**: 1144-1148.
- Ju J, Ruan C, Fuller CW, Glazer AN Mathies RA. (1995) Energy transfer fluorescent dye-labeled primers for DNA sequencing and analysis. *Proc. Natl. Acad. Sci. USA* **92**: 4347-4351.
- 10 Kamal A, Laxman E, Rao NV. (1999) A mild and rapid regeneration of alcohols from their allylic ethers by chlorotrimethylsilane/sodium iodide. *Tetrahedron letters* **40**: 371-372.
- Kheterpal I, Scherer J, Clark SM, Radhakrishnan A, Ju J, Ginther CL, Sensabaugh GF, Mathies RA. (1996) DNA Sequencing Using a Four-Color Confocal Fluorescence Capillary Array Scanner. *Electrophoresis.* **17**: 1852-1859.
- 15 Khoukhi N, Vaultier M, Carrie R. (1987) Synthesis and reactivity of methyl-azido butyrates and ethyl-azido valerates and of the corresponding acid chlorides as useful reagents for the aminoalkylation. *Tetrahedron* **43**: 1811-1822.
- Lee LG, Connell CR, Woo SL, Cheng RD, Mcardle BF, Fuller CW, Halloran ND, Wilson RK. (1992) DNA sequencing with dye-labeled terminators and T7 DNA-polymerase-effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucleic Acids Res.* **20**: 2471-2483.
- 20 Lee LG, Spurgeon SL, Heiner CR, Benson SC, Rosenblum BB, Menchen SM, Graham RJ, Constantinescu A, upadhya KG, Cassel JM, (1997) New energy transfer dyes for DNA sequencing. *Nucleic Acids Res.* **25**: 2816-2822.
- 25 Liu H.H., Felton C.,Xue Q.F.,Zhang B., Jedrzejewski P., Karger B.L. and Foret F. (2000) Development of multichannel Devices with an Array of Electrospray tips for high-throughput mass spectrometry. *Anal. Chem.* **72**:3303-3310.
- Metzker ML, Raghavachari R, Richards S, Jacutin SE, Civitello A, Burgess K, Gibbs RA. (1994) Termination of DNA synthesis by novel 3'-modified-deoxyribonucleoside 5'-triphosphates. *Nucleic Acids Res.* **22**: 4259-4267.
- 30 O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S, Fornace AJ Jr, Kohn KW. (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res.* **57**: 4285-4300.
- 35 Olejnik J, Ludemann HC, Krzymanska-Olejnik E, Berkenkamp S, Hillenkamp F, Rothschild KJ. (1999) Photocleavable peptide-DNA conjugates: synthesis and applications to DNA analysis using MALDI-MS. *Nucleic Acids Res.* **27**: 4626-4631.
- 40 Olejnik J, Sonar S, Krzymanska-Olejnik E, Rothschild KJ. (1995) Photocleavable biotin derivatives: a versatile approach for the isolation of biomolecules. *Proc. Natl. Acad. Sci. USA.* **92**: 7590-7594.
- Pelletier H, Sawaya MR, Kumar A, Wilson SH, Kraut J. (1994) Structures of ternary complexes of rat DNA polymerase β , a DNA template-primer, and ddCTP. *Science* **264**: 1891-1903.
- 45 Pennisi E. (2000) DOE Team Sequences Three Chromosomes. *Science* **288**: 417 - 419.
- Pillai VNR. (1980) Photoremovable Protecting Groups in Organic Synthesis. *Synthesis* 1-62.
- 50 Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. (1987) A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* **238**: 336-341.
- 55 Rollaf F. (1982) Sodium-borohydride reactions under phase-transfer conditions - reduction of azides to amines. *J. Org. Chem.* **47**: 4327-4329.
- Ronaghi M, Uhlen M, Nyren P. (1998) A sequencing Method based on real-time pyrophosphate. *Science* **281**:

364-365.

Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, Chen SM. (1997) New dye-labeled terminators for improved DNA sequencing patterns. *Nucleic Acids Res.* **25**: 4500-4504.

Roses A. (2000) Pharmacogenetics and the practice of medicine. *Nature.* **405**: 857-865.

Salas-Solano O, Carrilho E, Kotler L, Miller AW, Goetzinger W, Sosic Z, Karger BL, (1998) Routine DNA sequencing of 1000 bases in less than one hour by capillary electrophoresis with replaceable linear polyacrylamide solutions. *Anal. Chem.* **70**: 3996-4003.

Saxon E and Bertozzi CR (2000) Cell surface engineering by a modified Staudinger reaction. *Science* **287**: 2007-2010.

Schena M, Shalon D, Davis, R. Brown P.O. (1995) Quantitative monitoring of gene expression patterns with a cDNA microarray. *Science* **270**: 467-470.

Simpson PC, Adam DR, Woolley T, Thorsen T, Johnston R, Sensabaugh GF, and Mathies RA. (1998) High-throughput genetic analysis using microfabricated 96-sample capillary array electrophoresis microplates. *Proc. Natl. Acad. Sci. U. S. A.* **95**: 2256-2261.

Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell CR, Heiner C, Kent SBH, Hood LE. (1986) Fluorescence detection in automated DNA sequencing analysis. *Nature* **321**: 674-679.

Tabor S, Richardson C.C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 4767-4771.

Tabor S. & Richardson, CC. (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 6339-6343.

Turro NJ. (1991) Modern Molecular Photochemistry; University Science Books, Mill Valley, CA.

Velculescu VE, Zhang, I, Vogelstein, B. and Kinzler KW (1995) Serial Analysis of Gene Expression. *Science* **270**: 484-487.

Welch MB, Burgess K, (1999) Synthesis of fluorescent, photolabile 3'-O-protected nucleoside triphosphates for the base addition sequencing scheme. *Nucleosides and Nucleotides* **18**: 197-201.

Woolley AT, Mathies RA. (1994) Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. *Proc. Natl. Acad. Sci. USA.* **91**: 11348-11352.

Woolley AT, Sensabaugh GF and Mathies RA. (1997) High-Speed DNA Genotyping Using Microfabricated Capillary Array Electrophoresis Chips, *Anal. Chem.* **69**(11): 2181-2186.

Yamakawa H, Ohara O. (1997) A DNA cycle sequencing reaction that minimizes compressions on automated fluorescent sequencers. *Nucleic Acids Res.* **25**: 1311-1312.

Zhang XH, Chiang VL, (1996) Single-stranded DNA ligation by T4 RNA ligase for PCR cloning of 5'-noncoding fragments and coding sequence of a specific gene. *Nucleic Acids Res.* **24**: 990-991.

Zhang B., Liu H. Karger BL. Foret F. (1999) Microfabricated devices for capillary electrophoresis-electrospray mass spectrometry. *Anal. Chem.* **71**: 3258-3264.

Zhu Z, Chao J, Yu H, Waggoner AS. (1994) Directly labeled DNA probes using fluorescent nucleotides with different length linkers. *Nucleic Acids Res.* **22**: 3418-3422.

SEQUENCE LISTING

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Claims

- 50 1. A method for sequencing a nucleic acid by sequentially determining the identity of nucleotide analogues after the nucleotide analogues are incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:
- 55 (i) attaching a 5' end of the nucleic acid to a solid surface;
 (ii) attaching a primer to the nucleic acid attached to the solid surface;
 (iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from

the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is -CH₂OCH₃ or -CH₂CH=CH₂;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) determining the identity of the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the -OH group at the 3'-position of the deoxyribose to uncap the -OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (vii) so as to determine, for each repetition, the identity of the newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order in which steps (v) through (vii) are performed is: (v), (vi) and (vii); and if the unique label is a mass tag, the order in which steps (v) through (vii) are performed is: (vi), (vii) and (v).

2. The method of claim 1, wherein the solid surface is glass, silicon, or gold.

3. The method of claim 1, wherein the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip.

4. The method of claim 1, wherein the step of attaching the nucleic acid to the solid surface comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to the 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

5. The method of claim 4, wherein the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

6. The method of claim 1, wherein the nucleic acid that is attached to the solid surface is a single-stranded DNA.

7. The method of claim 1, wherein the nucleic acid that is attached to the solid surface in step (i) is a double-stranded DNA, wherein only one strand is directly attached to the solid surface, and wherein the strand that is not directly attached to the solid surface is removed by denaturing before proceeding to step (ii).

8. The method of claim 1, wherein the nucleic acid that is attached to the solid surface is a RNA, and the polymerase in step (iii) is reverse transcriptase.

9. The method of claim 1, wherein the primer is attached to a 3' end of the nucleic acid in step (ii) and wherein the attached primer comprises a stable loop and an -OH group at a 3'-position of a deoxyribose capable of self-priming in the polymerase reaction.

10. The method of claim 1, wherein the step of attaching the primer to the nucleic acid comprises hybridizing the primer to the nucleic acid or ligating the primer to the nucleic acid.

11. The method of claim 1, wherein one or more of four different nucleotide analogues is added in step (iii), wherein each different nucleotide analogue comprises a different base selected from the group consisting of thymine or uracil or an analogue of thymine or uracil, adenine or an analogue of adenine, cytosine or an analogue of cytosine, and guanine or an analogue of guanine, and wherein each of the four different nucleotide analogues comprises a unique label.

12. The method of claim 1, wherein the cleavable chemical group that caps the -OH group at the 3' -position of the deoxyribose in the nucleotide analogue is - CH₂CH=CH₂.
13. The method of claim 1, wherein the unique label that is attached to the nucleotide analogue is a fluorescent moiety or a fluorescent semiconductor crystal.
14. The method of claim 13, wherein the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-x-rhodamine.
15. The method of claim 1, wherein the unique label that is attached to the nucleotide analogue is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor.
16. The method of claim 15, wherein the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine.
17. The method of claim 1, wherein the unique label that is attached to the nucleotide analogue is a mass tag that can be detected and differentiated by a mass spectrometer.
18. The method of claim 17, wherein the mass tag is selected from the group consisting of a 2-nitro- α -methyl-benzyl group, a 2-nitro- α -methyl-3-fluorobenzyl group, a 2-nitro- α -methyl-3,4-difluorobenzyl group, and a 2-nitro- α -methyl-3,4-dimethoxybenzyl group.
19. The method of claim 1, wherein the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine.
20. The method of claim 1, wherein the cleavable linker between the unique label and the nucleotide analogue is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.
21. The method of claim 20, wherein the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.
22. The method of claim 1, wherein the cleavable chemical group used to cap the -OH group at the 3'-position of the deoxyribose is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.
23. The method of claim 1, wherein the chemical compounds added in step (vi) to permanently cap any unreacted -OH group on the primer attached to the nucleic acid or on the primer extension strand are a polymerase and one or more different dideoxynucleotides or analogues of dideoxynucleotides.
24. The method of claim 23, wherein the different dideoxynucleotides are selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine 5'-triphosphate, and their analogues.
25. The method of claim 1, wherein a polymerase and one or more of four different dideoxynucleotides are added in step (vi), and wherein each different dideoxynucleotide is selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate or an analogue of 2',3'-dideoxyadenosine 5'-triphosphate; 2',3'-dideoxyguanosine 5'-triphosphate or an analogue of 2',3'-dideoxyguanosine 5'-triphosphate; 2',3'-dideoxycytidine 5'-triphosphate or an analogue of 2',3'-dideoxycytidine 5'-triphosphate; and 2',3'-dideoxythymidine 5'-triphosphate or 2',3'-dideoxyuridine 5'-triphosphate or an analogue of 2',3'-dideoxythymidine 5'-triphosphate or an analogue of 2',3'-dideoxyuridine 5'-triphosphate.
26. The method of claim 17, wherein the mass tag is detected using a parallel mass spectrometry system which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.
27. A method of simultaneously sequencing a plurality of different nucleic acids, which comprises simultaneously applying

the method of claim 1 to the plurality of different nucleic acids.

28. Use of the method of claim 1 or 27 for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

29. A method of attaching a nucleic acid to a solid surface which comprises:

- (i) coating the solid surface with a phosphine moiety,
- (ii) attaching an azido group to a 5' end of the nucleic acid, and
- (iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

30. The method of claim 29, wherein the step of coating the solid surface with the phosphine moiety comprises:

- (i) coating the surface with a primary amine, and
- (ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

31. The method of claim 29, wherein the solid surface is glass, silicon, or gold.

32. The method of claim 29, wherein the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip.

33. The method of claim 29, wherein the nucleic acid that is attached to the solid surface is a single-stranded DNA, a double-stranded DNA or a RNA.

34. The method of claim 33, wherein the nucleic acid is a double-stranded DNA and only one strand is attached to the solid surface.

35. The method of claim 34, wherein the strand of the double-stranded DNA that is not attached to the solid surface is removed by denaturing.

36. Use of the method of claim 29 for attaching a nucleic acid to a solid surface for gene expression analysis, microarray based gene expression analysis, mutation detection, translational analysis, or transcriptional analysis of said nucleic acid.

37. A nucleotide analogue which comprises:

- (a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;
- (b) a unique label attached through a cleavable linker to the base or to an analogue of the base;
- (c) a deoxyribose; and
- (d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose, wherein the cleavable chemical group is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

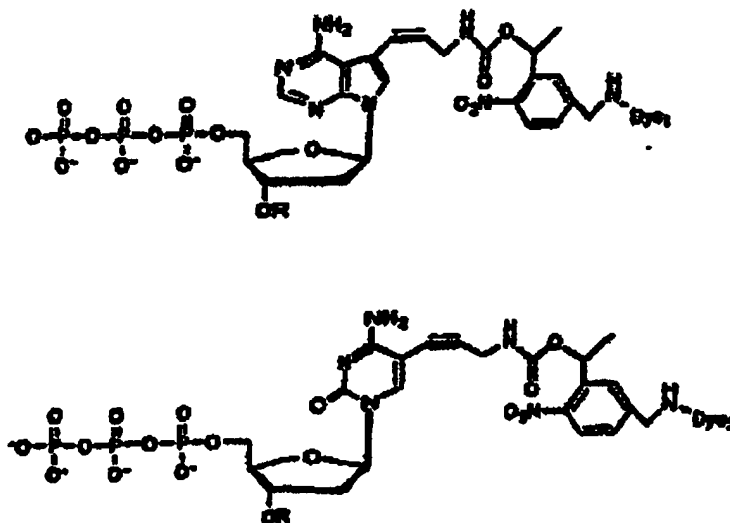
38. The nucleotide analogue of claim 37, wherein the cleavable chemical group that caps the -OH group at the 3'-position of the deoxyribose in the nucleotide analogue is $-\text{CH}_2\text{CH}=\text{CH}_2$.

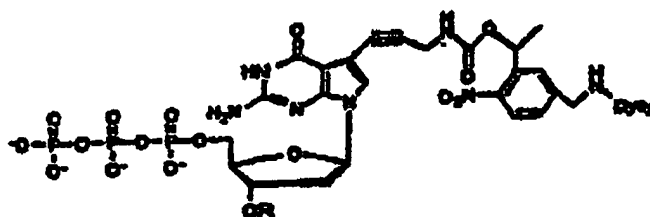
39. The nucleotide analogue of claim 37, wherein the unique label is a fluorescent moiety or a fluorescent semiconductor crystal.

40. The nucleotide analogue of claim 39, wherein the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine.

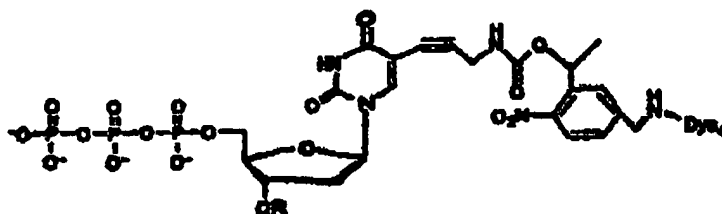
41. The nucleotide analogue of claim 37, wherein the unique label is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor.

42. The nucleotide analogue of claim 41, wherein the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocaroxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine.
43. The nucleotide analogue of claim 37, wherein the unique label is a mass tag that can be detected and differentiated by a mass spectrometer.
44. The nucleotide analogue of claim 43, wherein the mass tag is selected from the group consisting of a 2-nitro- α -methyl-benzyl group, a 2-nitro- α -methyl-3-fluorobenzyl group, a 2-nitro- α -methyl-3,4-difluorobenzyl group, and a 2-nitro- α -methyl-3,4-dimethoxybenzyl group.
45. The nucleotide analogue of claim 37, wherein the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine.
46. The nucleotide analogue of claim 37, wherein the linker between the unique label and the nucleotide analogue is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.
47. The nucleotide analogue of claim 46, wherein the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.
48. The nucleotide analogue of claim 37, wherein the cleavable chemical group used to cap the -OH group at the 3'-position of the deoxyribose is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.
49. The nucleotide analogue of claim 37, wherein the nucleotide analogue is selected from the group consisting of:



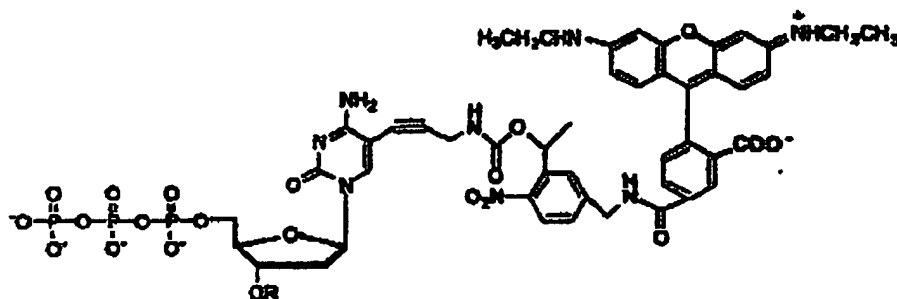
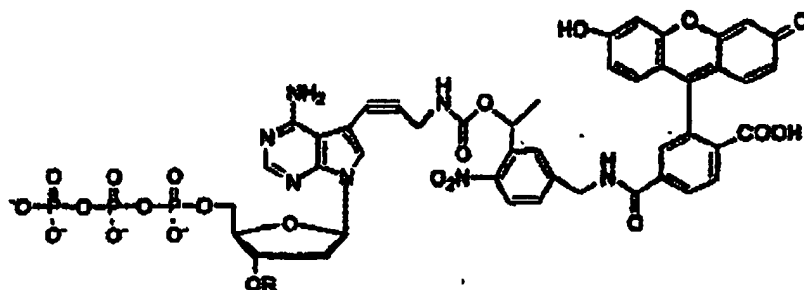


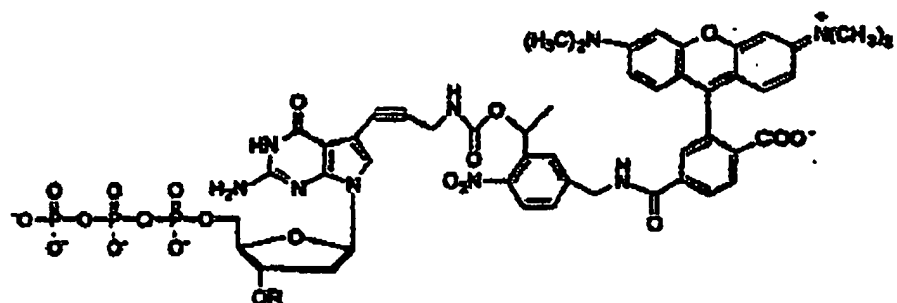
and



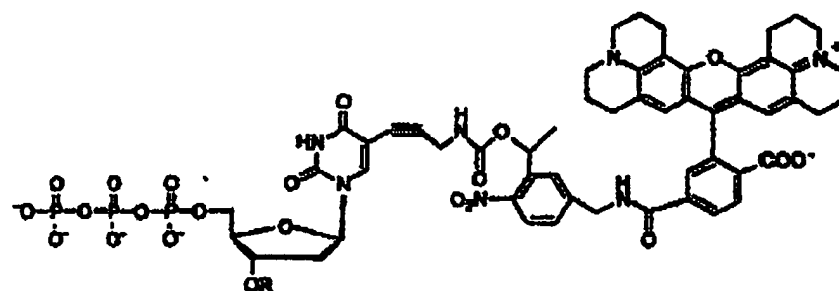
wherein Dye₁, Dye₂, Dye₃, and Dye₄ are four different dye labels; and
wherein R is -CH₂OCH₃ or -CH₂CH=CH₂.

50. The nucleotide analogue of claim 49, wherein the nucleotide analogue is selected from the group consisting of:



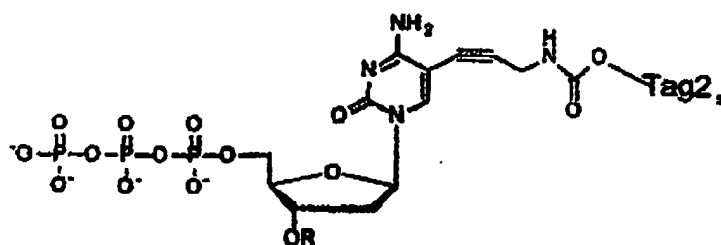
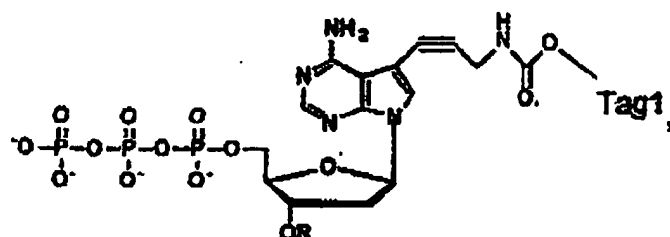


and



wherein R is -CH₂OCH₃ or -CH₂CH=CH₂.

51. The nucleotide analogue of claim 37, wherein the nucleotide analogue is selected from the group consisting of:



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and

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wherein Tag₁, Tag₂, Tag₃, and Tag₄ are four different mass tag labels; and
wherein R is -CH₂OCH₃ or -CH₂CH=CH₂.

52. The nucleotide analogue of claim 51, wherein the nucleotide analogue is selected from the group consisting of:

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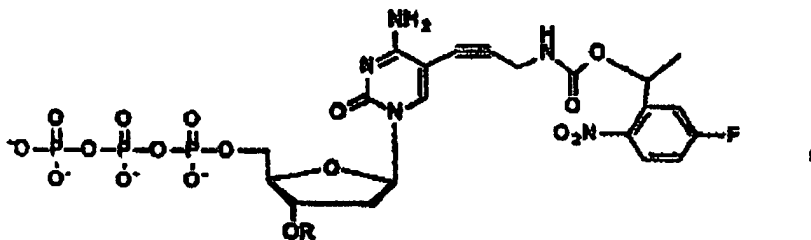
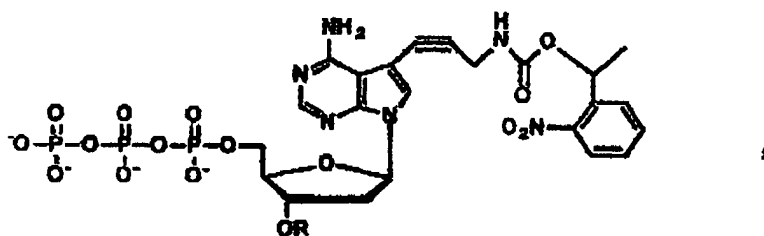
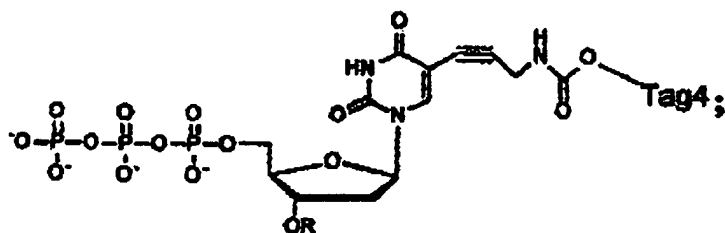
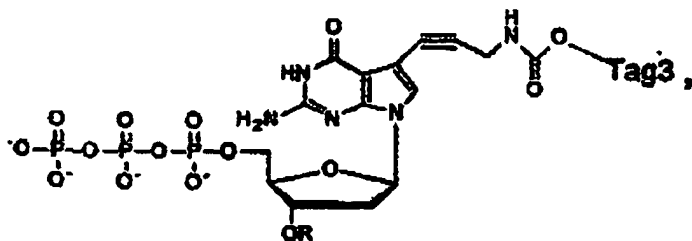
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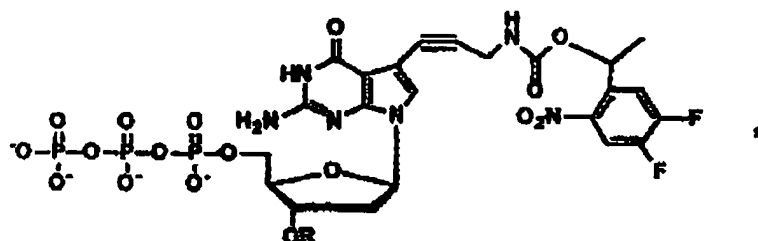
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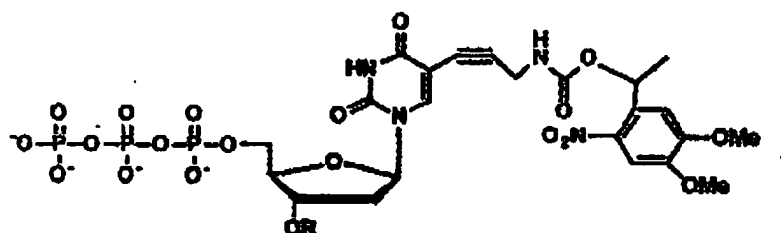
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and



wherein R is $-\text{CH}_2\text{OCH}_3$ or $-\text{CH}_2\text{CH}=\text{CH}_2$.

53. Use of the nucleotide analogue of claim 37 for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

Patentansprüche

1. Verfahren zum Sequenzieren einer Nukleinsäure durch sequentielles Bestimmen der Identität von Nukleotidanaloga nachdem die Nukleotidanaloga in einen wachsenden Strang von DNA eingebaut sind, in einer Polymerasereaktion, die die folgenden Schritte umfasst:

- (i) Anheften eines 5' Endes der Nukleinsäure an eine feste Oberfläche;
- (ii) Anheften eines Primers an die an die feste Oberfläche angeheftete Nukleinsäure;
- (iii) Zugabe einer Polymerase und eines oder mehrerer verschiedener Nukleotidanaloga zu der Nukleinsäure um **dadurch** ein Nukleotidanalogen in den wachsenden Strang von DNA einzubauen, wobei das eingebaute Nukleotidanalogen die Polymerasereaktion beendet, und wobei jedes verschiedene Nukleotidanalogen umfasst
 - (a) eine Base ausgewählt aus der Gruppe bestehend aus Adenin, Guanin, Cytosin, Thymin, und Uracil, und ihrer Analoga;
 - (b) eine eindeutige Markierung anheftet über einen spaltbaren Linker an die Base oder an ein Analogon der Base;
 - (c) eine Desoxyribose; und
 - (d) eine spaltbare chemische Gruppe um eine -OH-Gruppe an einer 3'-Position der Desoxyribose zu schützen, wobei die spaltbare chemische Gruppe $-\text{CH}_2\text{OCH}_3$ oder $-\text{CH}_2\text{CH}=\text{CH}_2$ ist;
- (iv) Waschen der festen Oberfläche, um nicht-ingebaute Nukleotidanaloga zu entfernen;
- (v) Bestimmen der Identität der eindeutigen Markierung, angehängt an das Nukleotidanalogen, das in den wachsenden Strang von DNA eingebaut worden ist, um **dadurch** das eingebaute Nukleotidanalogen zu identifizieren;
- (vi) Zugabe einer oder mehrere chemischer Verbindungen, um dauerhaft jede nicht-reagierte -OH-Gruppe auf dem Primer, angehängt an die Nukleinsäure oder auf einem Primer-Extension-Strang, gebildet durch Zugabe eines oder mehrerer Nukleotide oder Nukleotidanaloga zu dem Primer zu schützen;
- (vii) Spalten des spaltbaren Linkers zwischen dem Nukleotidanalogen, das in den wachsenden Strang von DNA eingebaut worden ist, und der eindeutigen Markierung;
- (viii) Spalten der spaltbaren chemischen Gruppe, die die -OH-Gruppe an der 3'-Position der Desoxyribose schützt, um die -OH-Gruppe zu entschützen, und Waschen der festen Oberfläche um gespaltene Verbindungen

zu entfernen; und

(ix) Wiederholen der Schritte (iii) bis (viii), um für jede Wiederholung, die Identität des neu eingebauten Nukleotidanalogs in den wachsenden Strang von DNA zu bestimmen;

- 5 wobei, wenn die eindeutige Markierung ein Farbstoff ist, die Reihenfolge, in welcher die Schritte (v) bis (vii) durchgeführt werden: (v), (vi) und (vii) ist; und
wenn die eindeutige Markierung ein Massen-Tag ist, die Reihenfolge, in welcher die Schritte (v) bis (vii) durchgeführt werden: (vi), (vii) und (v) ist.
- 10 2. Verfahren nach Anspruch 1, wobei die feste Oberfläche Glas, Silikon oder Gold ist.
3. Verfahren nach Anspruch 1, wobei die feste Oberfläche ein Magnetkügelchen, ein Chip, ein Kanal in einem Chip oder ein poröser Kanal in einem Chip ist.
- 15 4. Verfahren nach Anspruch 1, wobei der Schritt des Anheftens der Nukleinsäure an die feste Oberfläche umfasst:
 - (i) Beschichten der festen Oberfläche mit einem Phosphinrest,
 - (ii) Anheften einer Azidogruppe an das 5'-Ende der Nukleinsäure, und
 - (iii) Immobilisieren des 5'-Endes der Nukleinsäure auf der festen Oberfläche durch Wechselwirkung zwischen dem Phosphinrest auf der festen Oberfläche und der Azidogruppe am 5'-Ende der Nukleinsäure.
- 20 5. Verfahren nach Anspruch 4, wobei der Schritt des Beschichtens der festen Oberfläche mit dem Phosphinrest umfasst:
 - (i) Beschichten der Oberfläche mit einem primären Amin, und
 - (ii) Kovalentes Binden eines N-Hydroxysuccinimidylesters von Triarylphosphin mit dem primären Amin.
- 25 6. Verfahren nach Anspruch 1, wobei die Nukleinsäure, die an die feste Oberfläche angeheftet wird, eine einzelsträngige DNA ist.
- 30 7. Verfahren nach Anspruch 1, wobei die Nukleinsäure, die in Schritt (i) an die feste Oberfläche angeheftet wird, eine doppelsträngige DNA ist, wobei nur ein Strang direkt an die feste Oberfläche angeheftet wird, und wobei der Strang, der nicht direkt an die feste Oberfläche angeheftet wird, vor dem Übergang zu Schritt (ii) durch Denaturieren entfernt wird.
- 35 8. Verfahren nach Anspruch 1, wobei die Nukleinsäure, die an die feste Oberfläche angeheftet wird, eine RNA ist, und die Polymerase in Schritt (iii) reverse Transkriptase ist.
9. Verfahren nach Anspruch 1, wobei der Primer an ein 3'-Ende der Nukleinsäure in Schritt (ii) angeheftet wird, und wobei der angeheftete Primer eine stabile Schleife und eine -OH-Gruppe an einer 3'-Position einer Desoxyribose umfasst, fähig zum Selbstprimen in der Polymerasereaktion.
- 40 10. Verfahren nach Anspruch 1, wobei der Schritt des Anheftens des Primers an die Nukleinsäure Hybridisieren des Primers an die Nukleinsäure oder Ligieren des Primers an die Nukleinsäure umfasst.
- 45 11. Verfahren nach Anspruch 1, wobei eines oder mehrere von vier verschiedenen Nukleotidanaloga in Schritt (iii) zugegeben wird, wobei jedes verschiedene Nukleotidanalogen eine verschiedene Base umfasst, ausgewählt aus der Gruppe bestehend aus Thymin oder Uracil oder einem Analogon von Thymin oder Uracil, Adenin oder einem Analogon von Adenin, Cytosin oder einem Analogon von Cytosin, und Guanin oder einem Analogon von Guanin, und wobei jedes der vier verschiedenen Nukleotidanaloga eine eindeutige Markierung umfasst.
- 50 12. Verfahren nach Anspruch 1, wobei die spaltbare chemische Gruppe, die die -OH-Gruppe an der 3'-Position der Desoxyribose in dem Nukleotidanalogen schützt -CH₂CH=CH₂ ist.
13. Verfahren nach Anspruch 1, wobei die eindeutige Markierung, die an das Nukleotidanalogen angeheftet ist, ein fluoreszierender Rest oder ein fluoreszierender Halbleiterkristall ist.
- 55 14. Verfahren nach Anspruch 13, wobei der fluoreszierende Rest ausgewählt ist aus der Gruppe bestehend aus 5-Carboxyfluorescein, 6-Carboxyrhodamin-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamin, und 6-carboxy-X-rhoda-

min.

15. Verfahren nach Anspruch 1, wobei die eindeutige Markierung, die an das Nukleotidanalogen angeheftet ist, ein Fluoreszenzenergieübertragungs-Tag ist, die einen Energieübertragungsdonor und einen Energieübertragungsakzeptor umfasst.
16. Verfahren nach Anspruch 15, wobei der Energieübertragungsdonor 5-Carboxyfluorescein oder Cyanin ist, und wobei der Energieübertragungsakzeptor ausgewählt ist aus der Gruppe bestehend aus Dichlorcarboxyfluorescein, Dichlor-6-carboxyrhodamin-6G, Dichlor-N,N,N',N'-tetramethyl-6-carboxyrhodamin, und Dichlor-6-carboxy-X-rhodamin.
17. Verfahren nach Anspruch 1, wobei die eindeutige Markierung, die an das Nukleotidanalogen angeheftet ist, ein Massen-Tag ist, die über ein Massenspektrometer detektiert und unterschieden werden kann.
18. Verfahren nach Anspruch 17, wobei das Massen-Tag ausgewählt ist aus der Gruppe bestehend aus einer 2-nitro- α -methyl-benzyl-Gruppe, einer 2-nitro- α -methyl-3-fluorbenzyl-Gruppe, einer 2-nitro- α -methyl-3,4-difluorbenzyl-Gruppe, und einer 2-nitro- α -methyl-3,4-dimethoxybenzyl-Gruppe.
19. Verfahren nach Anspruch 1, wobei die eindeutige Markierung über einen spaltbaren Linker an eine 5-Position von Cytosin oder Thymin oder an eine 7-Position von Deaza-adenin oder Deaza-guanin angeheftet ist.
20. Verfahren nach Anspruch 1, wobei der spaltbare Linker zwischen der eindeutigen Markierung und dem Nukleotidanalogen gespalten wird durch ein Mittel ausgewählt aus der Gruppe bestehend aus einem oder mehreren physikalischen Mitteln, einem chemischen Mittel, einem physikalisch-chemischen Mittel, Hitze und Licht.
21. Verfahren nach Anspruch 20, wobei der spaltbare Linker ein photospaltbarer Linker ist, der einen 2-Nitrobenzylrest umfasst.
22. Verfahren nach Anspruch 1, wobei die spaltbare chemische Gruppe, verwendet um die -OH-Gruppe an der 3'-Position der Desoxyribose zu schützen, gespalten wird durch ein Mittel ausgewählt aus der Gruppe bestehend aus einem oder mehreren physikalischen Mitteln, einem chemischen Mittel, eines physikalisch-chemischen Mittel, Hitze und Licht.
23. Verfahren nach Anspruch 1, wobei die in Schritt (vi) zugegebenen chemischen Verbindungen zum permanenten Schützen jeder nicht-reagierten -OH-Gruppe auf dem Primer, angeheftet an die Nukleinsäure oder auf dem Primer-Extensionstrang, eine Polymerase und ein oder mehrere verschiedene Didesoxynukleotide oder Analoga von Didesoxynukleotiden sind.
24. Verfahren nach Anspruch 23, wobei die verschiedenen Didesoxynukleotide ausgewählt sind aus der Gruppe bestehend aus 2',3'-Didesoxyadenosin-5'-triphosphat, 2',3'-Didesoxyguanosin-5'-triphosphat, 2',3'-Didesoxycytidin-5'-triphosphat, 2',3'-Didesoxythymidin-5'-triphosphat, 2',3'-Didesoxyuridin-5'-triphosphat, und ihre Analoga.
25. Verfahren nach Anspruch 1, wobei eine Polymerase und ein oder mehrere von vier verschiedenen Didesoxynukleotiden in Schritt (vi) zugegeben werden, und wobei jedes verschiedene Didesoxynukleotid ausgewählt ist aus der Gruppe bestehend aus 2',3'-Didesoxyadenosin-5'-triphosphat oder einem Analogon von 2',3'-Didesoxyadenosin-5'-triphosphat; 2',3'-Didesoxyguanosin-5'-triphosphat oder einem Analogon von 2',3'-Didesoxyguanosin-5'-triphosphat; 2',3'-Didesoxycytidin-5'-triphosphat oder einem Analogon von 2',3'-Didesoxycytidin-5'-triphosphat; und 2',3'-Didesoxythymidin-5'-triphosphat oder 2',3'-Didesoxyuridin-5'-triphosphat oder einem Analogon von 2',3'-Didesoxythymidin-5'-triphosphat oder einem Analogon von 2',3'-Didesoxyuridin-5'-triphosphat.
26. Verfahren nach Anspruch 17, wobei das Massen-Tag detektiert wird unter Verwendung eines parallelen Massenspektrometrysystems, das eine Vielzahl von atmospheric pressure chemical ionization-Massenspektrometern umfasst zur parallelen Analyse einer Vielzahl von Proben, die Massen-Tags umfassen.
27. Ein Verfahren zum gleichzeitigen Sequenzieren einer Vielzahl von verschiedenen Nukleinsäuren, welches das gleichzeitige Anwenden des Verfahrens nach Anspruch 1 auf die Vielzahl von verschiedenen Nukleinsäuren umfasst.
28. Verwendung des Verfahrens nach Anspruch 1 oder 27 zum Nachweis von Einzelnukleotidpolymorphismen, genetische Mutationsanalyse, serielle Analyse von Genexpression, Genexpressionsanalyse, Identifikation in der Foren-

sik, Assoziationsstudien genetischer Krankheiten, DNA-Sequenzierung, genomische Sequenzierung, Translationsanalyse oder Transkriptionsanalyse.

29. Ein Verfahren zum Anheften einer Nukleinsäure an eine feste Oberfläche, welches umfasst:
 - (i) Beschichten der festen Oberfläche mit einem Phosphinrest,
 - (ii) Anheften einer Azidogruppe an ein 5'-Ende der Nukleinsäure, und
 - (iii) Immobilisieren des 5'-Endes der Nukleinsäure auf der festen Oberfläche durch Wechselwirkung zwischen dem Phosphinrest auf der festen Oberfläche und der Azidogruppe am 5'-Ende der Nukleinsäure.
30. Verfahren nach Anspruch 29, wobei der Schritt des Beschichtens der festen Oberfläche mit dem Phosphinrest umfasst:
 - (i) Beschichten der Oberfläche mit einem primären Amin, und
 - (ii) Kovalentes Binden eines N-Hydroxysuccinimidylesters von Triarylphosphin mit dem primären Amin.
31. Verfahren nach Anspruch 29, wobei die feste Oberfläche Glas, Silikon oder Gold ist.
32. Verfahren nach Anspruch 29, wobei die feste Oberfläche ein Magnetkügelchen, ein Chip, ein Kanal in einem Chip oder ein poröser Kanal in einem Chip ist.
33. Verfahren nach Anspruch 29, wobei die Nukleinsäure, die an die feste Oberfläche angeheftet wird, eine einzelsträngige DNA, eine doppelsträngige DNA oder eine RNA ist.
34. Verfahren nach Anspruch 33, wobei die Nukleinsäure eine doppelsträngige DNA ist und nur ein Strang an die feste Oberfläche angeheftet ist.
35. Verfahren nach Anspruch 34, wobei der Strang der doppelsträngigen DNA, der nicht an die feste Oberfläche angeheftet ist, durch Denaturieren entfernt wird.
36. Verwendung des Verfahrens nach Anspruch 29 zum Anheften einer Nukleinsäure an eine feste Oberfläche für Genexpressionsanalyse, Microarray-basierte Genexpressionsanalyse, Mutationsnachweis, Translationsanalyse, oder Transkriptionsanalyse dieser Nukleinsäure.
37. Ein Nukleotidanalogen welches umfasst:
 - (a) eine Base ausgewählt aus der Gruppe bestehend aus Adenin oder einem Analogon von Adenin, Cytosin oder einem Analogon von Cytosin, Guanin oder einem Analogon von Guanin, Thymin oder einem Analogon von Thymin, und Uracil oder einem Analogon von Uracil;
 - (b) eine eindeutige Markierung angeheftet über einen spaltbaren Linker an die Base oder an ein Analogon der Base;
 - (c) eine Desoxyribose; und
 - (d) eine spaltbare chemische Gruppe, um eine -OH-Gruppe an einer 3'-Position der Desoxyribose zu schützen, wobei die spaltbare chemische Gruppe $-\text{CH}_2\text{OCH}_3$ oder $-\text{CH}_2\text{CH}=\text{CH}_2$ ist.
38. Nukleotidanalogen nach Anspruch 37, wobei die spaltbare chemische Gruppe, welche die -OH-Gruppe an der 3'-Position der Desoxyribose in dem Nukleotidanalogen schützt $-\text{CH}_2\text{CH}=\text{CH}_2$ ist.
39. Nukleotidanalogen nach Anspruch 37, wobei die eindeutige Markierung ein fluoreszierender Rest oder ein fluoreszierender Halbleiterkristall ist.
40. Nukleotidanalogen nach Anspruch 39, wobei der fluoreszierende Rest ausgewählt ist aus der Gruppe bestehend aus 5-Carboxyfluorescein, 6-Carboxyrhodamin-6G, N,N,N',N'-Tetramethyl-6-carboxyrhodamin, und 6-Carboxy-X-rhodamin.
41. Nukleotidanalogen nach Anspruch 37, wobei die eindeutige Markierung ein Fluoreszenzenergieübertragungs-Tag ist, die einen Energieübertragungsdonor und einen Energieübertragungsakzeptor umfasst.

42. Nukleotidanalogen nach Anspruch 41, wobei der Energieübertragungsdonor 5-Carboxyfluorescein oder Cyanin ist, und wobei der Energieübertragungsakzeptor ausgewählt ist aus der Gruppe bestehend aus Dichlorcarboxyfluorescein, Dichlor-6-carboxyrhodamin-6G, Dichlor-N,N,N',N'-tetramethyl-6-carboxyrhodamin, und Dichlor-6-carboxy-X-rhodamin.

43. Nukleotidanalogen nach Anspruch 37, wobei die eindeutige Markierung ein Massen-Tag ist, die durch ein Massenspektrometer detektiert und unterschieden werden kann.

44. Nukleotidanalogen nach Anspruch 43, wobei das Massen-Tag ausgewählt ist aus der Gruppe bestehend aus einer 2-Nitro- α -methyl-benzyl-Gruppe, einer 2-Nitro- α -methyl-3-fluorbenzyl-Gruppe, einer 2-Nitro- α -methyl-3,4-difluorbenzyl-Gruppe, und einer 2-Nitro- α -methyl-3,4-dimethoxybenzyl-Gruppe.

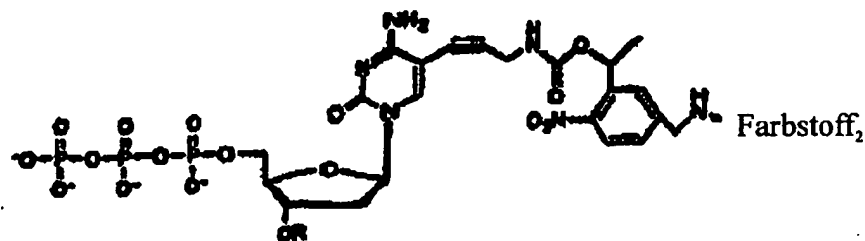
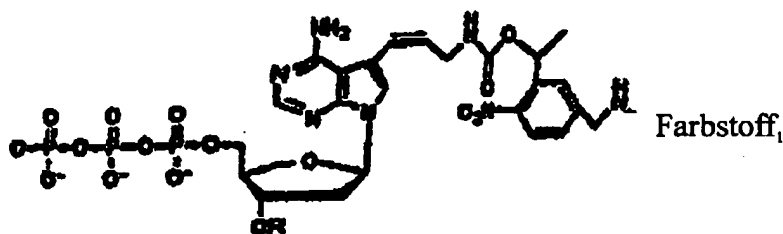
45. Nukleotidanalogen nach Anspruch 37, wobei die eindeutige Markierung über einen spaltbaren Linker an eine 5-Position von Cytosin oder Thymin oder an eine 7-Position von Deaza-adenin oder Deaza-guanin angeheftet ist.

46. Nukleotidanalogen nach Anspruch 37, wobei der Linker zwischen der eindeutigen Markierung und dem Nukleotidanalogen spaltbar ist durch ein Mittel ausgewählt aus der Gruppe bestehend aus einem oder mehreren physikalischen Mitteln, einem chemischen Mittel, einem physikalisch-chemischen Mittel, Hitze und Licht.

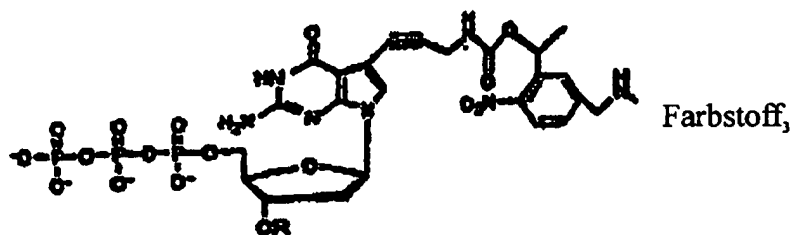
47. Nukleotidanalogen nach Anspruch 46, wobei der spaltbare Linker ein photospaltbarer Linker ist, der einen 2-Nitrobenzylrest umfasst.

48. Nukleotidanalogen nach Anspruch 37, wobei die spaltbare chemische Gruppe, verwendet um die -OH-Gruppe an der 3'-Position der Desoxyribose zu schützen, spaltbar ist durch ein Mittel ausgewählt aus der Gruppe bestehend aus einem oder mehreren physikalischen Mitteln, einem chemischen Mittel, einem physikalisch-chemischen Mittel, Hitze und Licht.

49. Nukleotidanalogen nach Anspruch 37, wobei das Nukleotidanalogen ausgewählt ist aus der Gruppe bestehend aus:



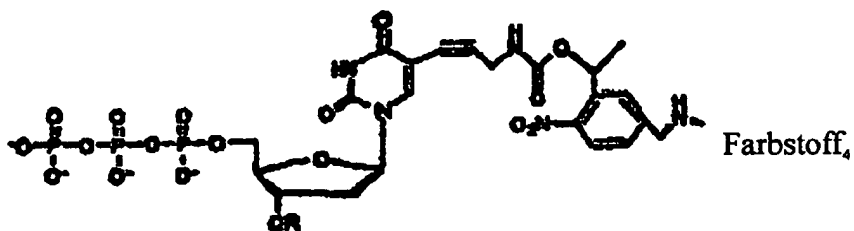
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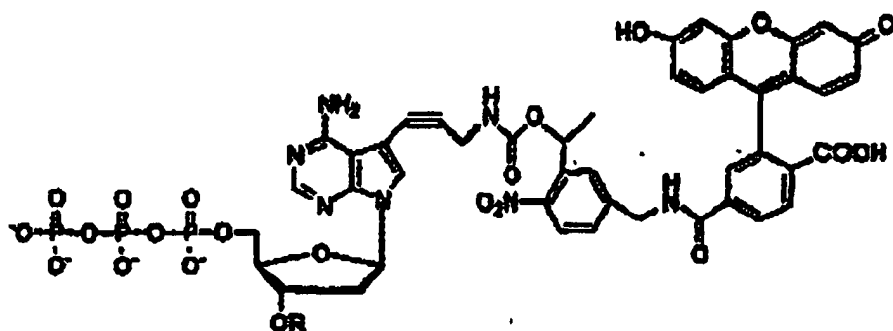
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wobei Farbstoff₁, Farbstoff₂, Farbstoff₃ und Farbstoff₄ vier verschiedene Farbstoffmarkierungen sind; und wobei R -CH₂OCH₃ oder -CH₂CH=CH₂ ist.

50. Nukleotidanalogen nach Anspruch 49, wobei das Nukleotidanalogen ausgewählt ist aus der Gruppe bestehend aus:

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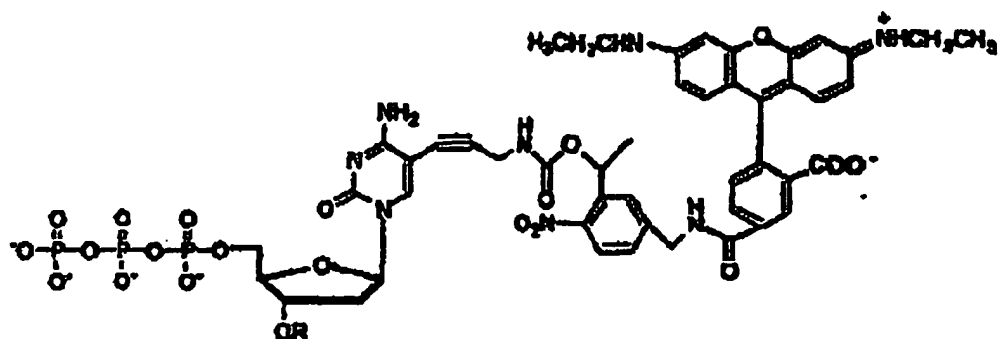
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wobei R -CH₂OCH₃ oder -CH₂CH=CH₂ ist.

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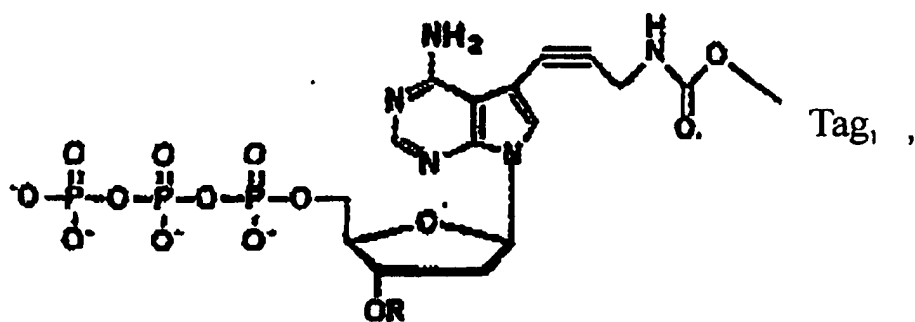
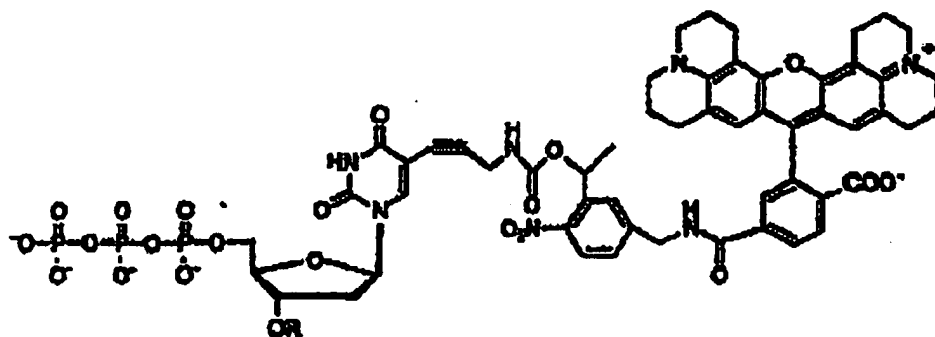
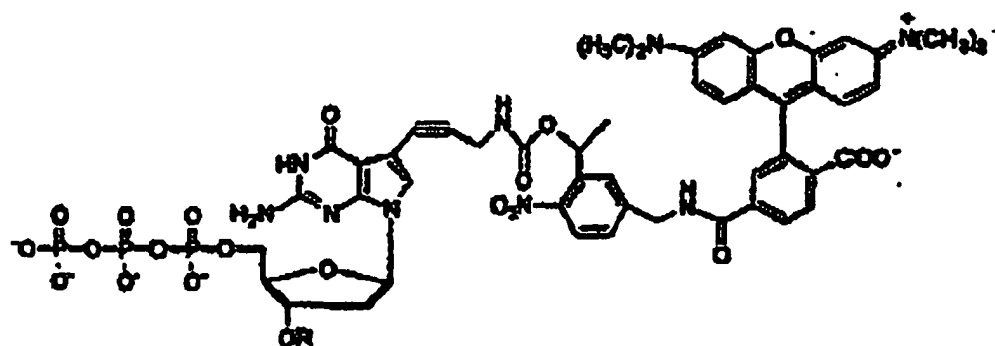
51. Nukleotidanalogen nach Anspruch 37, wobei das Nukleotidanalogen ausgewählt ist aus der Gruppe bestehend aus:

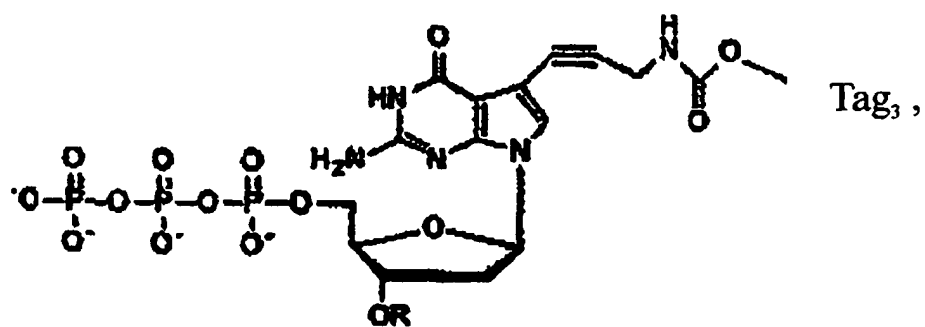
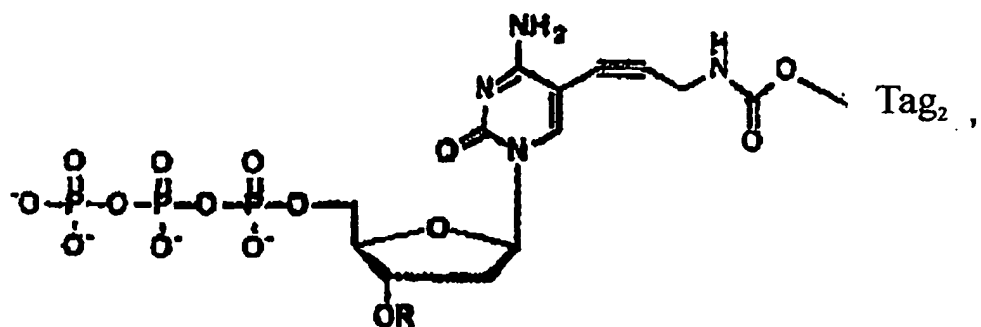
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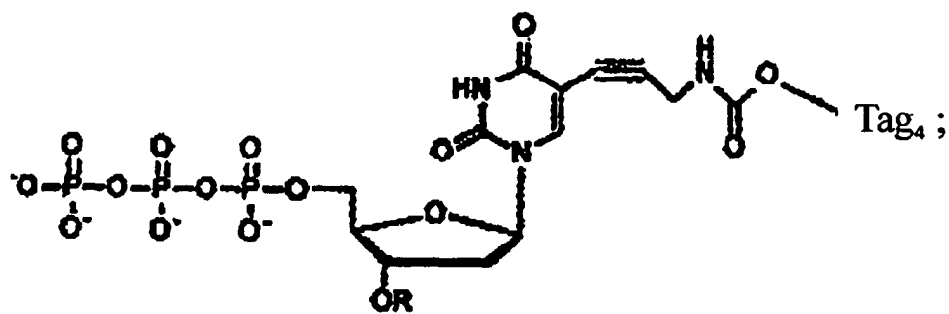
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und



wobei Tag_1 , Tag_2 , Tag_3 , und Tag_4 vier verschiedene Massen-Tag-Markierungen sind; und
wobei R $-\text{CH}_2\text{OCH}_3$ oder $-\text{CH}_2\text{CH}=\text{CH}_2$ ist.

50 **52.** Nukleotidanalogen nach Anspruch 51, wobei das Nukleotidanalogen ausgewählt ist aus der Gruppe bestehend aus:

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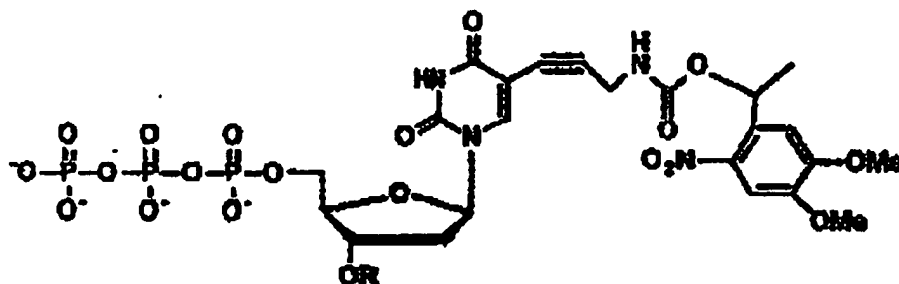
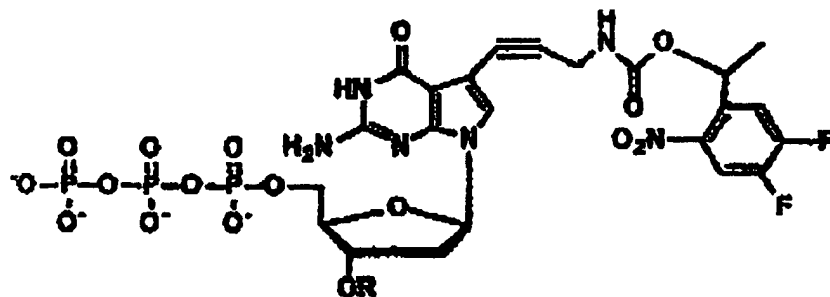
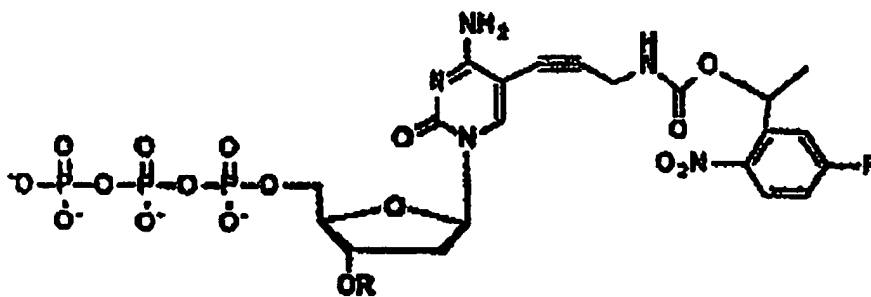
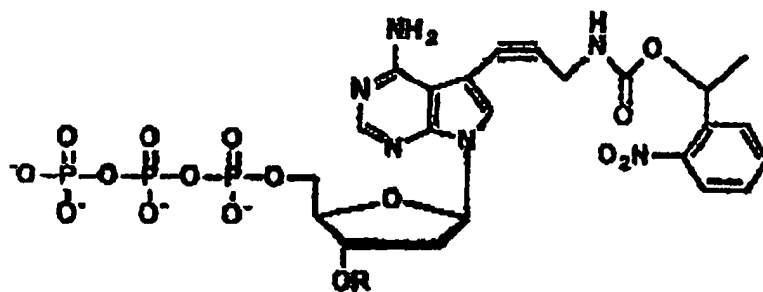
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und

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wobei R -CH₂OCH₃ oder -CH₂CH=CH₂ ist.

53. Verwendung des Nukleotidanalogs nach Anspruch 37 zum Nachweis von Einzelnukleotidpolymorphismen, genetische Mutationsanalyse, serielle Analyse von Genexpression, Genexpressionsanalyse, Identifizierung in der Forensik, Assoziationsstudien genetischer Krankheiten, DNA-Sequenzierung, genomische Sequenzierung, Translationsanalyse oder Transkriptionsanalyse.

5

Revendications

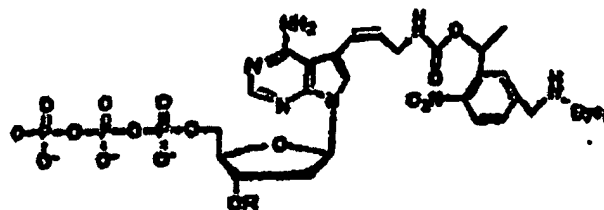
- 10 1. Procédé de séquençage d'un acide nucléique par détermination séquentielle de l'identité d'analogues nucléotidiques après que les analogues nucléotidiques ont été incorporés dans un brin en croissance d'ADN dans une réaction de polymérase, qui comprend les étapes suivantes :
- 15 (i) attacher une extrémité 5' de l'acide nucléique à une surface solide ;
 (ii) attacher une amorce à l'acide nucléique attaché à la surface solide ;
 (iii) ajouter une polymérase et un ou plusieurs analogues nucléotidiques différents à l'acide nucléique pour incorporer ainsi un analogue nucléotidique dans le brin en croissance d'ADN, dans lequel l'analogue nucléotidique incorporé interrompt la réaction de polymérase et dans lequel chaque analogue nucléotidique différent comprend (a) une base choisie dans le groupe constitué par l'adénine, la guanine, la cytosine, la thymine et l'uracile, et leurs analogues ; (b) un marqueur unique attaché par le biais d'un lieu clivable à la base ou à un analogue de la base ; (c) un désoxyribose ; et (d) un groupe chimique clivable pour coiffer un groupe -OH à une position 3' du désoxyribose, dans lequel le groupe chimique clivable est $-CH_2OCH_3$ ou $-CH_2CH=CH_2$;
 20 (iv) laver la surface solide pour retirer les analogues nucléotidiques non incorporés ;
 (v) déterminer l'identité du marqueur unique attaché à l'analogue nucléotidique qui a été incorporé dans le brin en croissance d'ADN, de manière à identifier ainsi les analogues nucléotidiques incorporés ;
 25 (vi) ajouter un ou plusieurs composés chimiques pour coiffer en permanence tout groupe -OH n'ayant pas réagi sur l'amorce attachée à l'acide nucléique ou sur un brin d'extension de l'amorce formé en ajoutant un ou plusieurs nucléotides ou analogues nucléotidiques à l'amorce ;
 (vii) cliver le lieu clivable entre l'analogue nucléotidique qui a été incorporé dans le brin en croissance d'ADN et le marqueur unique ;
 30 (viii) cliver le groupe chimique clivable coiffant le groupe -OH à la position 3' du désoxyribose pour décoiffer le groupe -OH, et laver la surface solide pour retirer les composés clivés ; et
 (ix) répéter les étapes (iii) à (viii) de manière à déterminer, pour chaque répétition, l'identité de l'analogue nucléotidique nouvellement incorporé dans le brin en croissance d'ADN ;
- 35 dans lequel, si le marqueur unique est un colorant, l'ordre dans lequel les étapes (v) à (vii) sont effectuées est : (v), (vi) et (vii) ; et
 si le marqueur unique est un marqueur de masse, l'ordre dans lequel les étapes (v) à (vii) sont effectuées est : (vi), (vii) et (v).
- 40 2. Procédé selon la revendication 1, dans lequel la surface solide est du verre, du silicium ou de l'or.
3. Procédé selon la revendication 1, dans lequel la surface solide est une bille magnétique, une puce, un canal dans une puce, ou un canal poreux dans une puce.
- 45 4. Procédé selon la revendication 1, dans lequel l'étape d'attachement de l'acide nucléique à la surface solide comprend :
- (i) le revêtement de la surface solide avec un groupe fonctionnel phosphine,
 50 (ii) l'attachement d'un groupe azido à l'extrémité 5' de l'acide nucléique, et
 (iii) l'immobilisation de l'extrémité 5' de l'acide nucléique sur la surface solide par interaction entre le groupe fonctionnel phosphine sur la surface solide et le groupe azido sur l'extrémité 5' de l'acide nucléique.
5. Procédé selon la revendication 4, dans lequel l'étape de revêtement de la surface solide avec le groupe fonctionnel phosphine comprend :
- 55 (i) le revêtement de la surface avec une amine primaire, et
 (ii) le couplage covalent d'un ester N-hydroxy-succinimidylique de triarylphosphine avec l'amine primaire.

6. Procédé selon la revendication 1, dans lequel l'acide nucléique qui est attaché à la surface solide est un ADN simple brin.
- 5 7. Procédé selon la revendication 1, dans lequel l'acide nucléique qui est attaché à la surface solide dans l'étape (i) est un ADN double brin, dans lequel un seul brin est directement attaché à la surface solide, et dans lequel le brin qui n'est pas directement attaché à la surface solide est retiré par dénaturation avant de passer à l'étape (ii).
8. Procédé selon la revendication 1, dans lequel l'acide nucléique qui est attaché à la surface solide est un ARN, et la polymérase dans l'étape (iii) est une transcriptase inverse.
- 10 9. Procédé selon la revendication 1, dans lequel l'amorce est attachée à une extrémité 3' de l'acide nucléique dans l'étape (ii) et dans lequel l'amorce attachée comprend une boucle stable et un groupe -OH à une position 3' d'un désoxyribose susceptible d'auto-amorçage dans la réaction de polymérase.
- 15 10. Procédé selon la revendication 1, dans lequel l'étape d'attachement de l'amorce à l'acide nucléique comprend l'hybridation de l'amorce avec l'acide nucléique ou la ligature de l'amorce à l'acide nucléique.
11. Procédé selon la revendication 1, dans lequel l'un ou plusieurs de quatre analogues nucléotidiques différents sont ajoutés dans l'étape (iii), dans lequel chaque analogue nucléotidique différent comprend une base différente choisie dans le groupe constitué par la thymine ou l'uracile ou un analogue de thymine ou d'uracile, l'adénine ou un analogue d'adénine, la cytosine ou un analogue de cytosine, et la guanine ou un analogue de guanine, et dans lequel chacun des quatre analogues nucléotidiques différents comprend un marqueur unique.
- 20 12. Procédé selon la revendication 1, dans lequel le groupe chimique clivable qui coiffe le groupe -OH à la position 3' du désoxyribose dans l'analogue nucléotidique est $-\text{CH}_2\text{CH}=\text{CH}_2$.
13. Procédé selon la revendication 1, dans lequel le marqueur unique qui est attaché à l'analogue nucléotidique est un groupe fonctionnel fluorescent ou un cristal semi-conducteur fluorescent.
- 30 14. Procédé selon la revendication 13, dans lequel le groupe fonctionnel fluorescent est choisi dans le groupe constitué par la 5-carboxyfluorescéine, la 6-carboxyrhodamine-6G, la N,N,N',N'-tétraméthyl-6-carboxyrhodamine et la 6-carboxy-X-rhodamine.
- 35 15. Procédé selon la revendication 1, dans lequel le marqueur unique qui est attaché à l'analogue nucléotidique est un marqueur de transfert d'énergie de fluorescence qui comprend un donneur de transfert d'énergie et un accepteur de transfert d'énergie.
16. Procédé selon la revendication 15, dans lequel le donneur de transfert d'énergie est la 5-carboxyfluorescéine ou la cyanine, et dans lequel l'accepteur de transfert d'énergie est choisi dans le groupe constitué par la dichlorocarboxyfluorescéine, la dichloro-6-carboxyrhodamine-6G, la dichloro-N,N,N',N'-tétraméthyl-6-carboxyrhodamine, et la dichloro-6-carboxy-X-rhodamine.
- 40 17. Procédé selon la revendication 1, dans lequel le marqueur unique qui est attaché à l'analogue nucléotidique est un marqueur de masse qui peut être détecté et différencié par un spectromètre de masse.
- 45 18. Procédé selon la revendication 17, dans lequel le marqueur de masse est choisi dans le groupe constitué par un groupe 2-nitro- α -méthyl-benzyle, un groupe 2-nitro- α -méthyl-3-fluorobenzyle, un groupe 2-nitro- α -méthyl-3,4-difluorobenzyle, et un groupe 2-nitro- α -méthyl-3,4-diméthoxybenzyle.
- 50 19. Procédé selon la revendication 1, dans lequel le marqueur unique est attaché par le biais d'un lien clivable à une position 5 d'une cytosine ou d'une thymine ou à une position 7 d'une déaza-adénine ou d'une déaza-guanine.
20. Procédé selon la revendication 1, dans lequel le lien clivable entre le marqueur unique et l'analogue nucléotidique est clivé par un moyen choisi dans le groupe constitué par l'un ou plusieurs parmi un moyen physique, un moyen chimique, un moyen physico-chimique, la chaleur, et la lumière.
- 55 21. Procédé selon la revendication 20, dans lequel le lien clivable est un lien photoclivable qui comprend un groupe fonctionnel 2-nitrobenzyle.

22. Procédé selon la revendication 1, dans lequel le groupe chimique clivable utilisé pour coiffer le groupe -OH à la position 3' du désoxyribose est clivé par un moyen choisi dans le groupe constitué par l'un ou plusieurs parmi un moyen physique, un moyen chimique, un moyen physico-chimique, la chaleur, et la lumière.
- 5 23. Procédé selon la revendication 1, dans lequel les composés chimiques ajoutés dans l'étape (vi) pour coiffer en permanence tout groupe -OH n'ayant pas réagi sur l'amorce attachée à l'acide nucléique ou sur le brin d'extension de l'amorce sont une polymérase et un ou plusieurs didésoxynucléotides ou analogues de didésoxynucléotides différents.
- 10 24. Procédé selon la revendication 23, dans lequel les didésoxynucléotides différents sont choisis dans le groupe constitué par la 2',3'-didésoxyadénosine 5'-triphosphate, la 2',3'-didésoxyguanosine 5'-triphosphate, la 2',3'-didésoxycytidine 5'-triphosphate, la 2',3'-didésoxythymidine 5'-triphosphate, la 2',3'-didésoxyuridine 5'-triphosphate, et leurs analogues.
- 15 25. Procédé selon la revendication 1, dans lequel une polymérase et l'un ou plusieurs de quatre didésoxynucléotides différents sont ajoutés dans l'étape (vi), et dans lequel chaque didésoxynucléotide différent est choisi dans le groupe constitué par la 2',3'-didésoxyadénosine 5'-triphosphate ou un analogue de 2',3'-didésoxyadénosine 5'-triphosphate ; la 2',3'-didésoxyguanosine 5'-triphosphate ou un analogue de 2',3'-didésoxyguanosine 5'-triphosphate ; la 2',3'-didésoxycytidine 5'-triphosphate ou un analogue de 2',3'-didésoxycytidine 5'-triphosphate ; et la 2',3'-didésoxythymidine 5'-triphosphate ou la 2',3'-didésoxyuridine 5'-triphosphate ou un analogue de 2',3'-didésoxythymidine 5'-triphosphate ou un analogue de 2',3'-didésoxyuridine 5'-triphosphate.
- 20 26. Procédé selon la revendication 17, dans lequel le marqueur de masse est détecté en utilisant un système de spectrométrie de masse parallèle qui comprend une pluralité de spectromètres de masse à ionisation chimique à pression atmosphérique pour analyse parallèle d'une pluralité d'échantillons comprenant des marqueurs de masse.
- 25 27. Procédé de séquençage simultané d'une pluralité d'acides nucléiques différents, qui comprend l'application simultanée du procédé selon la revendication 1 à la pluralité d'acides nucléiques différents.
- 30 28. Utilisation du procédé selon la revendication 1 ou 27 pour la détection de polymorphismes de nucléotides uniques, l'analyse de mutations génétiques, l'analyse en série de l'expression de gènes, l'analyse de l'expression de gènes, l'identification dans des expertises médico-légales, les études d'association de maladies génétiques, le séquençage d'ADN, le séquençage génomique, l'analyse traductionnelle, ou l'analyse transcriptionnelle.
- 35 29. Procédé d'attachement d'un acide nucléique à une surface solide qui comprend :
- (i) le revêtement de la surface solide avec un groupe fonctionnel phosphine,
- (ii) l'attachement d'un groupe azido à l'extrémité 5' de l'acide nucléique, et
- (iii) l'immobilisation de l'extrémité 5' de l'acide nucléique sur la surface solide par interaction entre le groupe fonctionnel phosphine sur la surface solide et le groupe azido sur l'extrémité 5' de l'acide nucléique.
- 40 30. Procédé selon la revendication 29, dans lequel l'étape de revêtement de la surface solide avec le groupe fonctionnel phosphine comprend :
- (i) le revêtement de la surface avec une amine primaire, et
- (ii) le couplage covalent d'un ester N-hydroxy-succinimidylique de triarylsphosphine avec l'amine primaire.
- 45 31. Procédé selon la revendication 29, dans lequel la surface solide est du verre, du silicium ou de l'or.
- 50 32. Procédé selon la revendication 29, dans lequel la surface solide est une bille magnétique, une puce, un canal dans une puce, ou un canal poreux dans une puce.
33. Procédé selon la revendication 29, dans lequel l'acide nucléique qui est attaché à la surface solide est un ADN simple brin, un ADN double brin ou un ARN.
- 55 34. Procédé selon la revendication 33, dans lequel l'acide nucléique est un ADN double brin et un seul brin est attaché à la surface solide.

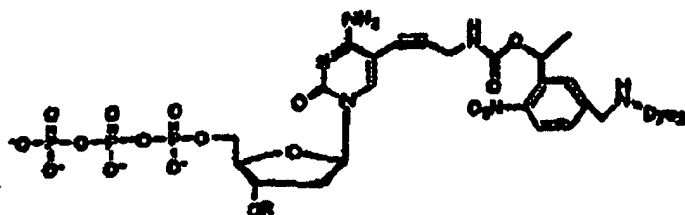
35. Procédé selon la revendication 34, dans lequel le brin de l'ADN double brin qui n'est pas attaché à la surface solide est retiré par dénaturation.
36. Utilisation du procédé selon la revendication 29 pour attacher un acide nucléique à une surface solide pour analyse de l'expression de gènes, analyse de l'expression de gènes basée sur des puces à ADN, détection de mutations, analyse traductionnelle, ou analyse transcriptionnelle dudit acide nucléique.
37. Analogue nucléotidique qui comprend :
- (a) une base choisie dans le groupe constitué par l'adénine ou un analogue d'adénine, la cytosine ou un analogue de cytosine, la guanine ou un analogue de guanine, la thymine ou un analogue de thymine, et l'uracile ou un analogue d'uracile ;
 - (b) un marqueur unique attaché par le biais d'un lien clivable à la base ou à un analogue de la base ;
 - (c) un désoxyribose ; et
 - (d) un groupe chimique clivable pour coiffer un groupe -OH à une position 3' du désoxyribose, dans lequel le groupe chimique clivable est $-\text{CH}_2\text{OCH}_3$ ou $-\text{CH}_2\text{CH}=\text{CH}_2$.
38. Analogue nucléotidique selon la revendication 37, dans lequel le groupe chimique clivable qui coiffe le groupe -OH à la position 3' du désoxyribose dans l'analogue nucléotidique est $-\text{CH}_2\text{CH}=\text{CH}_2$.
39. Analogue nucléotidique selon la revendication 37, dans lequel le marqueur unique est un groupe fonctionnel fluorescent ou un cristal semi-conducteur fluorescent.
40. Analogue nucléotidique selon la revendication 39, dans lequel le groupe fonctionnel fluorescent est choisi dans le groupe constitué par la 5-carboxyfluorescéine, la 6-carboxyrhodamine-6G, la N,N,N',N'-tétraméthyl-6-carboxyrhodamine, et la 6-carboxy-X-rhodamine.
41. Analogue nucléotidique selon la revendication 37, dans lequel le marqueur unique est un marqueur de transfert d'énergie de fluorescence qui comprend un donneur de transfert d'énergie et un accepteur de transfert d'énergie.
42. Analogue nucléotidique selon la revendication 41, dans lequel le donneur de transfert d'énergie est la 5-carboxyfluorescéine ou la cyanine, et dans lequel l'accepteur de transfert d'énergie est choisi dans le groupe constitué par la dichlorocarboxyfluorescéine, la dichloro-6-carboxyrhodamine-6G, la dichloro-N,N,N',N'-tétraméthyl-6-carboxyrhodamine, et la dichloro-6-carboxy-X-rhodamine.
43. Analogue nucléotidique selon la revendication 37, dans lequel le marqueur unique est un marqueur de masse qui peut être détecté et différencié par un spectromètre de masse.
44. Analogue nucléotidique selon la revendication 43, dans lequel le marqueur de masse est choisi dans le groupe constitué par un groupe 2-nitro- α -méthyl-benzyle, un groupe 2-nitro- α -méthyl-3-fluorobenzyle, un groupe 2-nitro- α -méthyl-3,4-difluorobenzyle, et un groupe 2-nitro- α -méthyl-3,4-diméthoxybenzyle.
45. Analogue nucléotidique selon la revendication 37, dans lequel le marqueur unique est attaché par le biais d'un lien clivable à une position 5 d'une cytosine ou d'une thymine ou à une position 7 d'une déaza-adénine ou d'une déaza-guanine.
46. Analogue nucléotidique selon la revendication 37, dans lequel le lien entre le marqueur unique et l'analogue nucléotidique est clivable par un moyen choisi dans le groupe constitué par l'un ou plusieurs parmi un moyen physique, un moyen chimique, un moyen physico-chimique, la chaleur, et la lumière.
47. Analogue nucléotidique selon la revendication 46, dans lequel le lien clivable est un lien photoclivable qui comprend un groupe fonctionnel 2-nitrobenzyle.
48. Analogue nucléotidique selon la revendication 37, dans lequel le groupe chimique clivable utilisé pour coiffer le groupe -OH à la position 3' du désoxyribose est clivable par un moyen choisi dans le groupe constitué par l'un ou plusieurs parmi un moyen physique, un moyen chimique, un moyen physico-chimique, la chaleur, et la lumière.
49. Analogue nucléotidique selon la revendication 37, l'analogue nucléotidique étant choisi dans le groupe constitué par :

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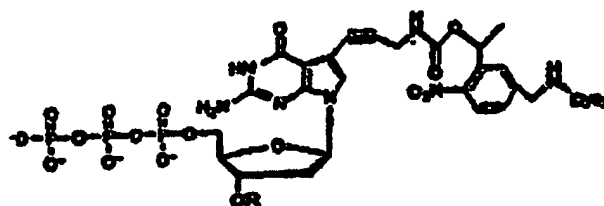
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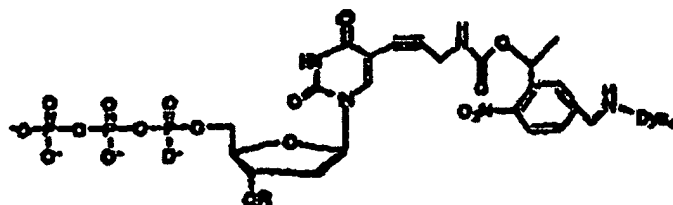
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et

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dans lesquels Dye₁, Dye₂, Dye₃ et Dye₄ sont quatre marqueurs colorants différents ; et
dans lesquels R est -CH₂OCH₃ ou -CH₂CH=CH₂.

50. Analogue nucléotidique selon la revendication 49, l'analogue nucléotidique étant choisi dans le groupe constitué par :

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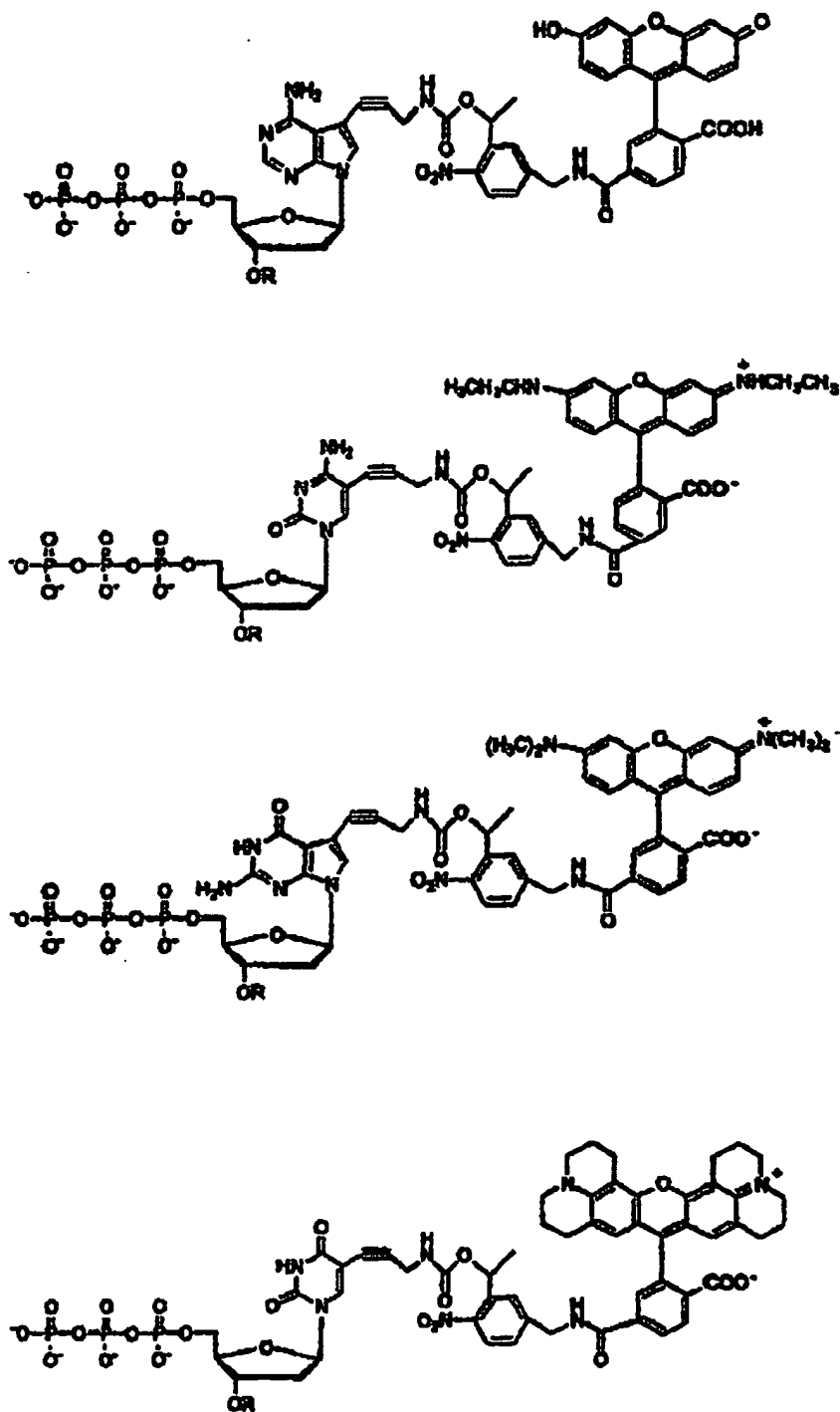
et

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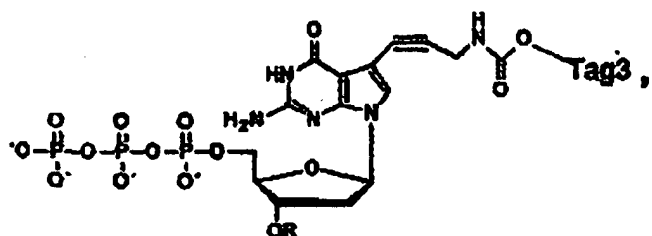
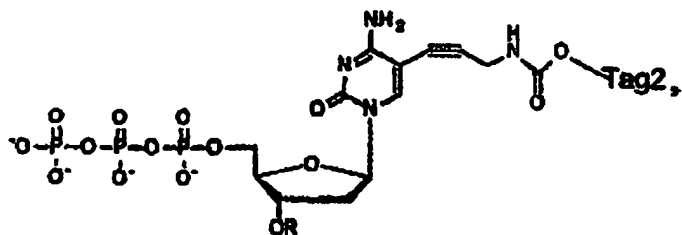
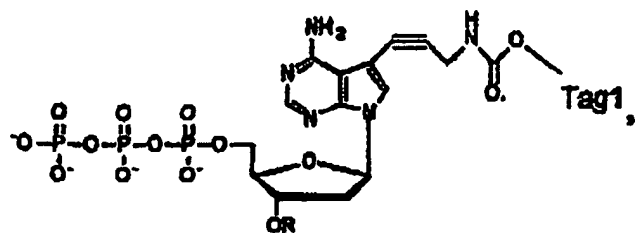
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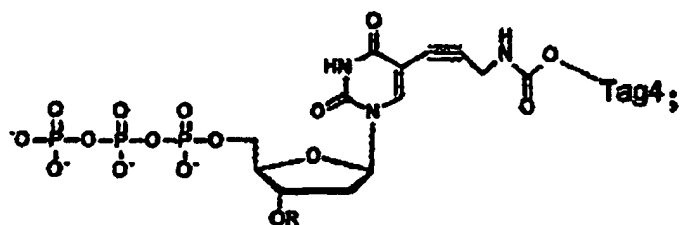


dans lesquels R est -CH₂OCH₃ ou -CH₂CH=CH₂.

51. Analogue nucléotidique selon la revendication 37, l'analogue nucléotidique étant choisi dans le groupe constitué par :

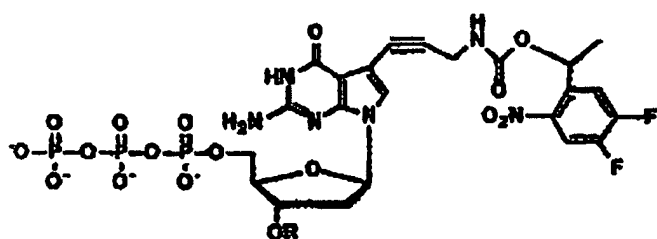
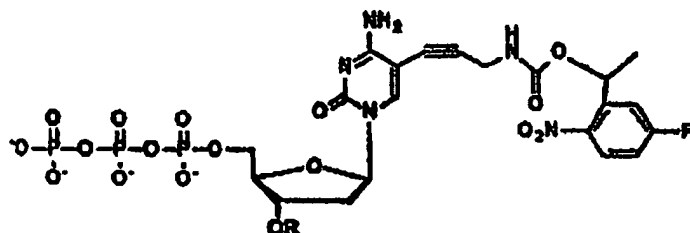
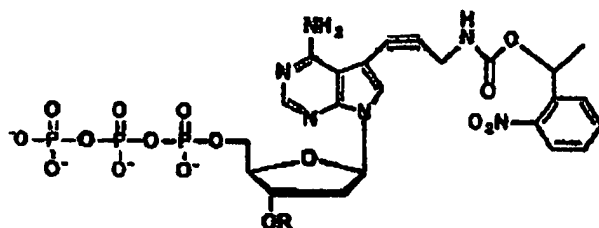


35 et

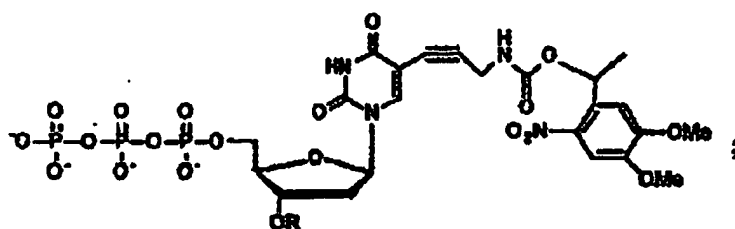


50 dans lesquels Tag₁, Tag₂, Tag₃ et Tag₄ sont quatre marqueurs de masse différents ; et dans lesquels R est -CH₂OCH₃ ou -CH₂CH=CH₂.

55 **52.** Analogue nucléotidique selon la revendication 51, l'analogue nucléotidique étant choisi dans le groupe constitué par :



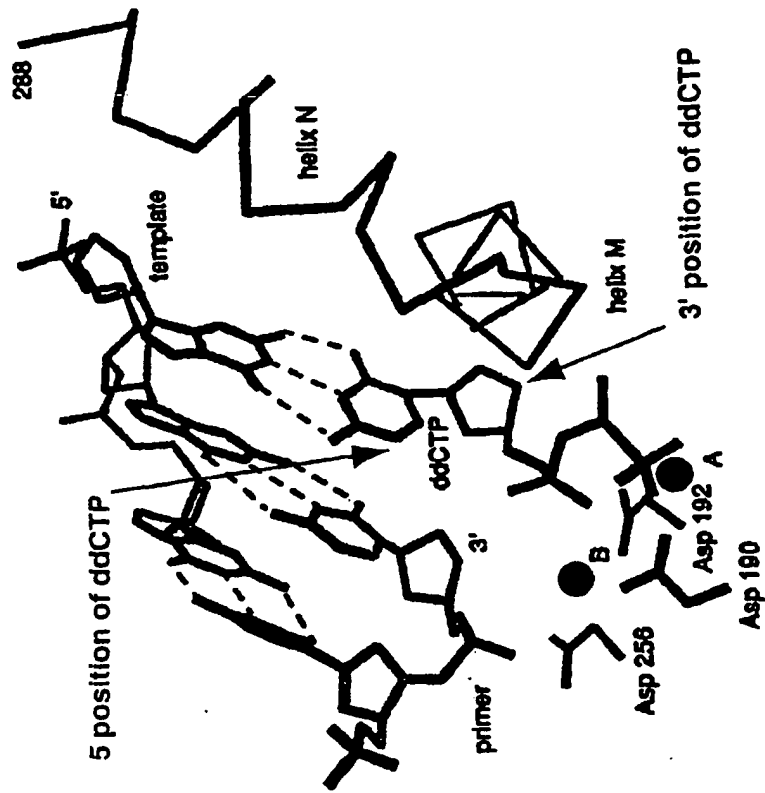
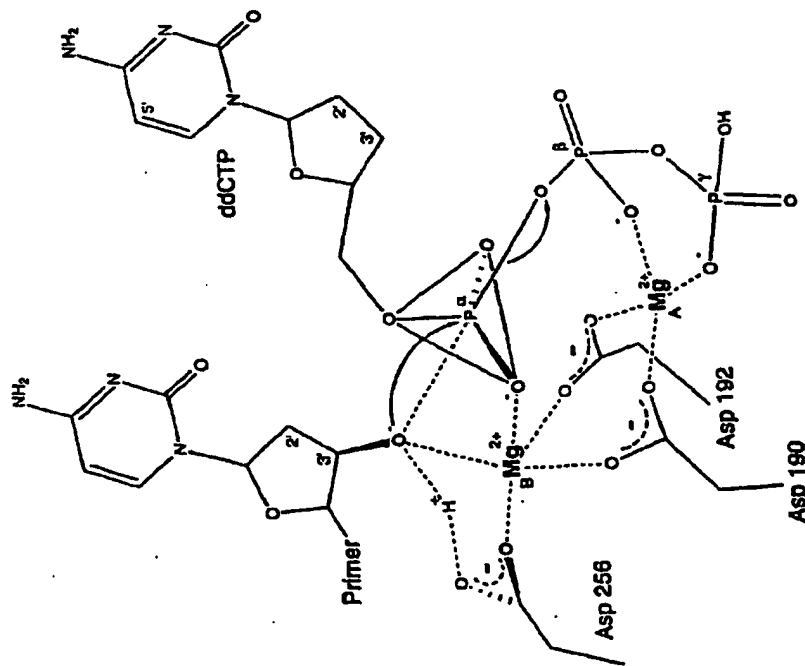
et



dans lesquels R est $-\text{CH}_2\text{OCH}_3$ ou $-\text{CH}_2\text{CH}=\text{CH}_2$.

53. Utilisation de l'analogue nucléotidique selon la revendication 37 pour la détection de polymorphismes de nucléotides uniques, l'analyse de mutations génétiques, l'analyse en série de l'expression de gènes, l'analyse de l'expression de gènes, l'identification dans des expertises médico-légales, les études d'association de maladies génétiques, le séquençage d'ADN, le séquençage génomique, l'analyse traductionnelle, ou l'analyse transcriptionnelle.

FIGURE 1



Asp = Aspartic Acid

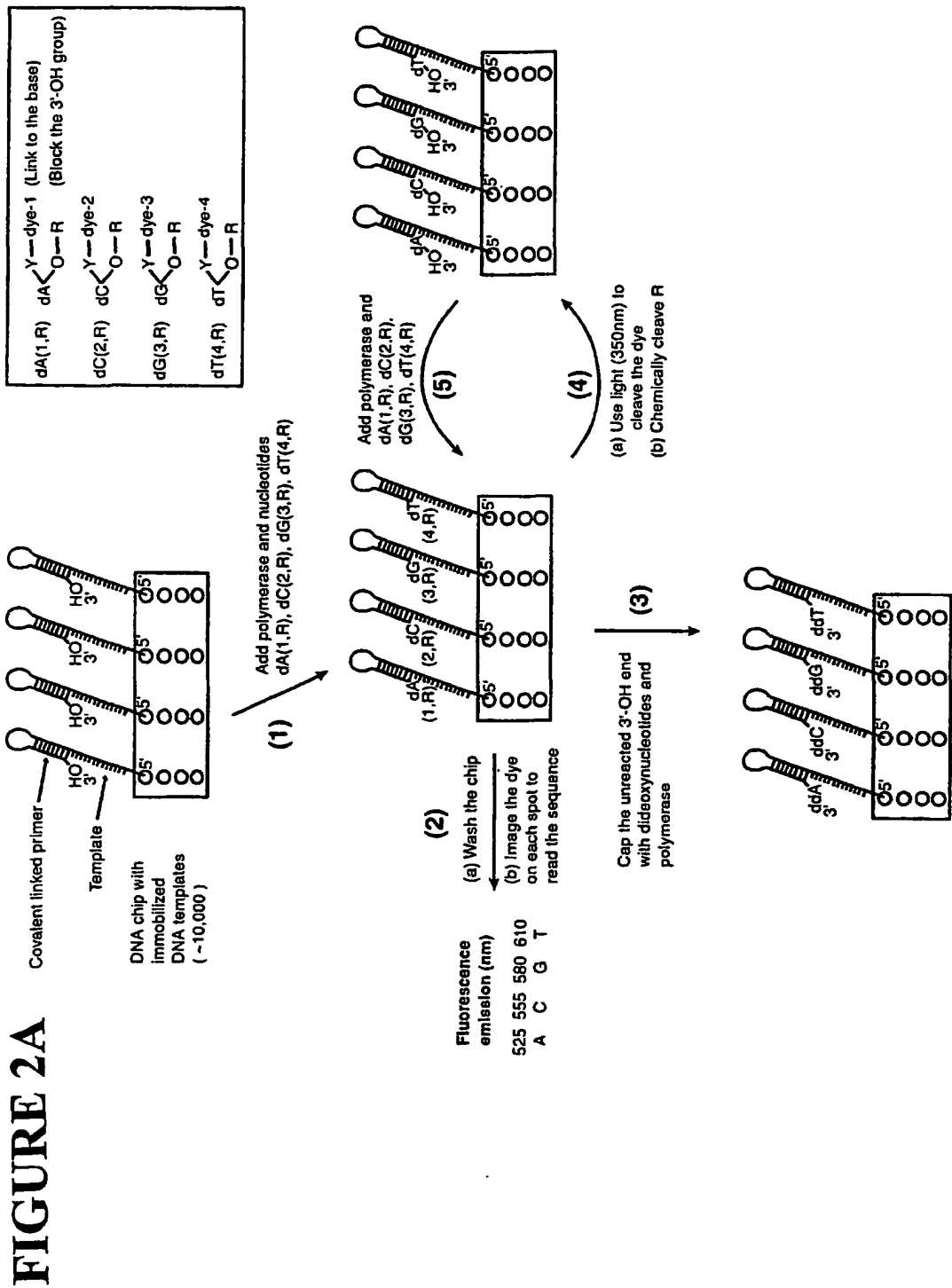


FIGURE 2B

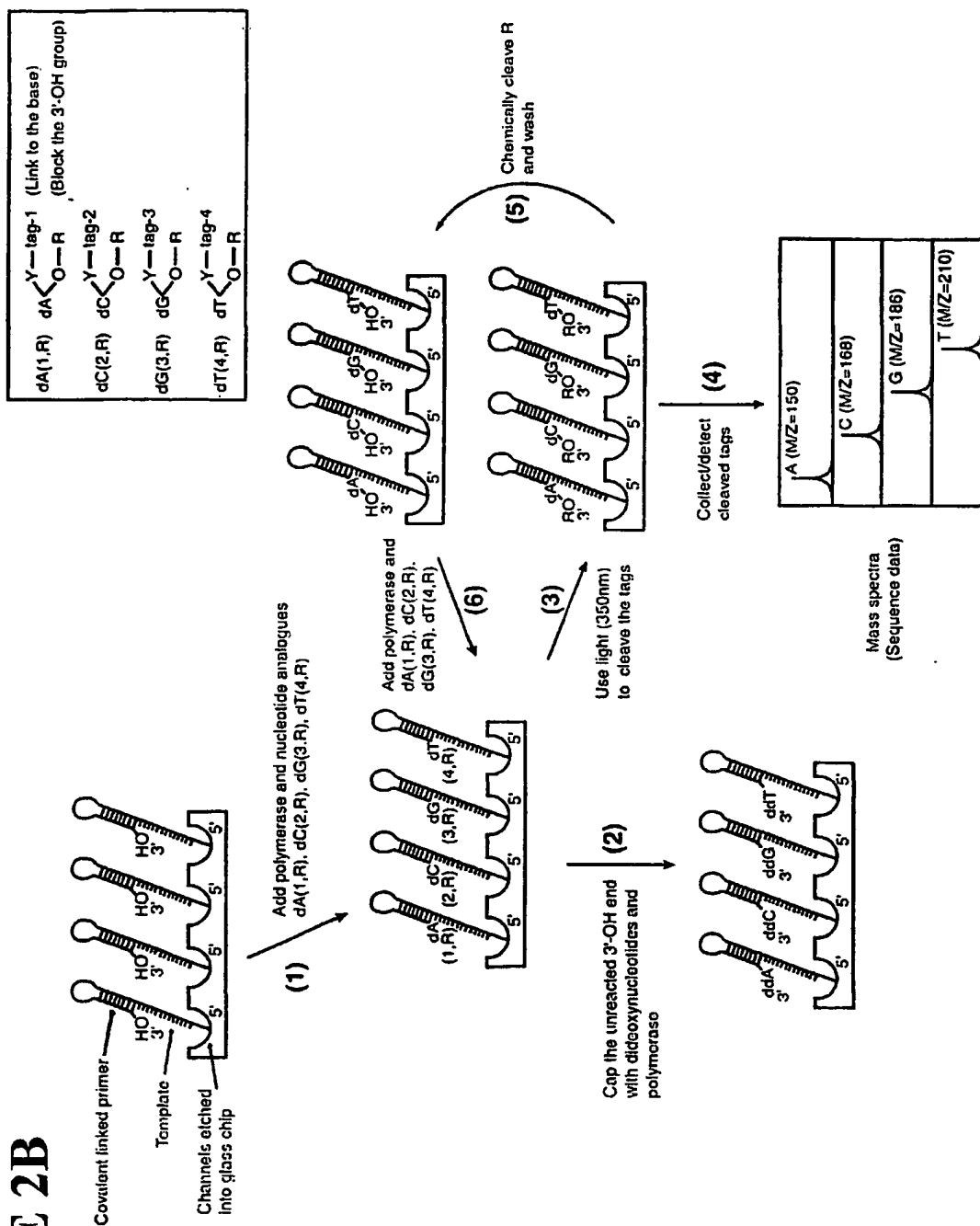


FIGURE 3

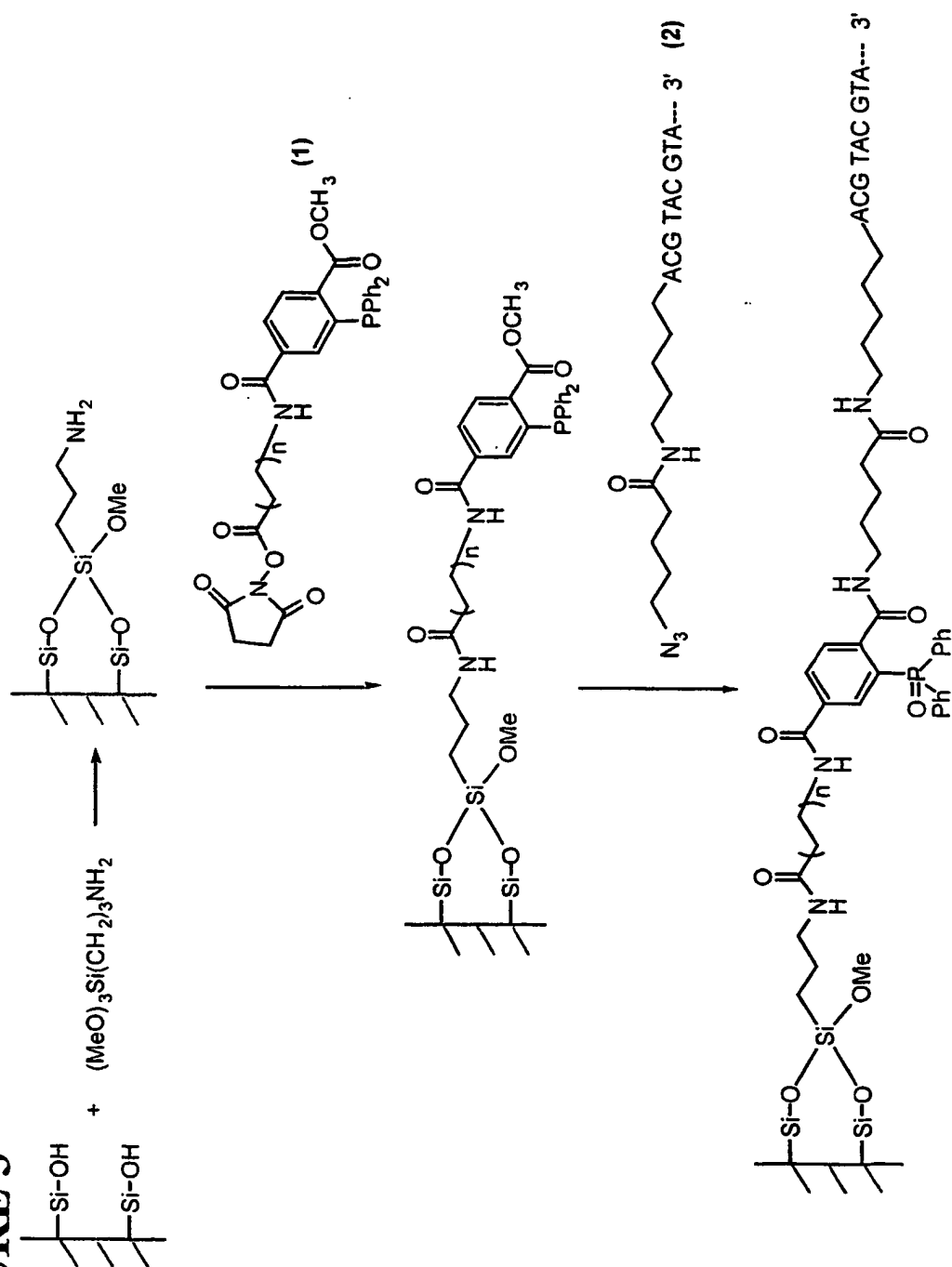
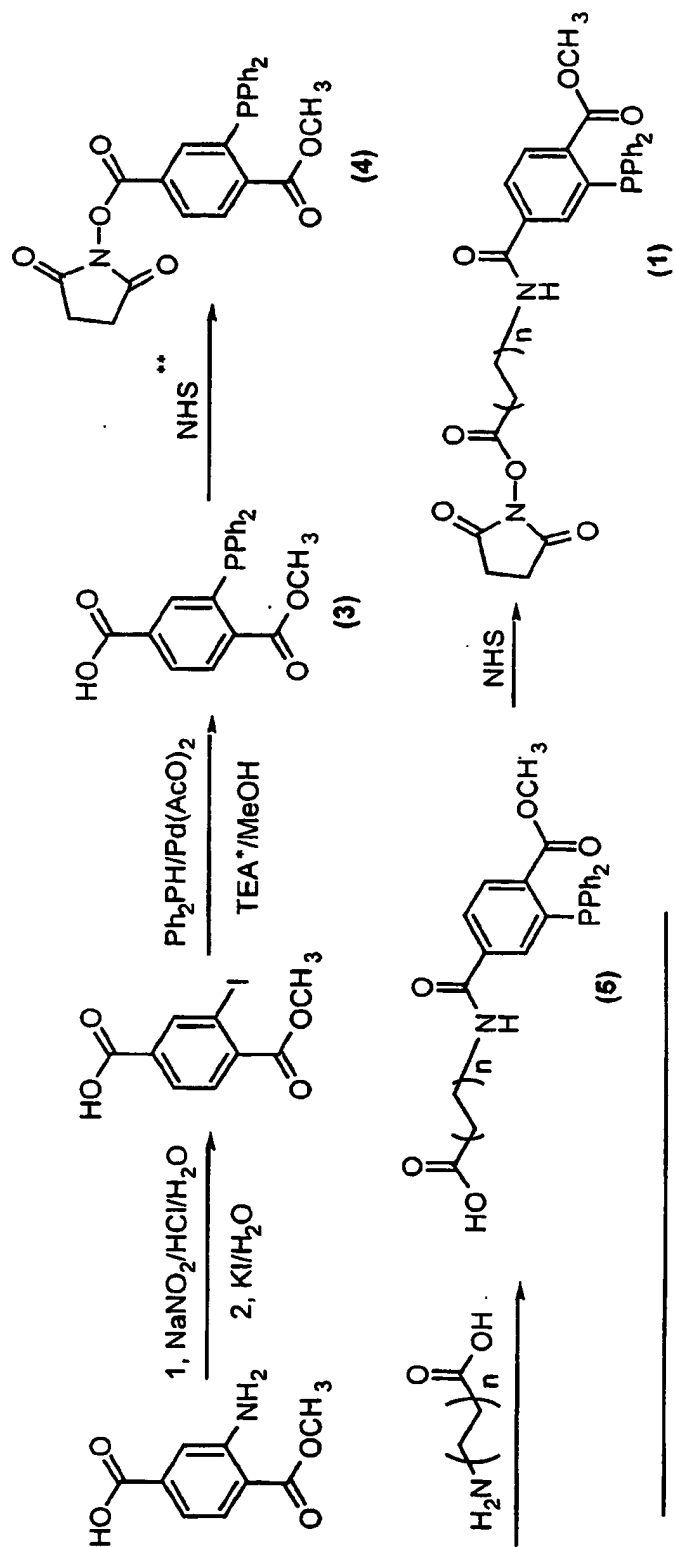


FIGURE 4



***TEA = Triethylamine,** NHS = N-Hydroxysuccinimide**

FIGURE 5

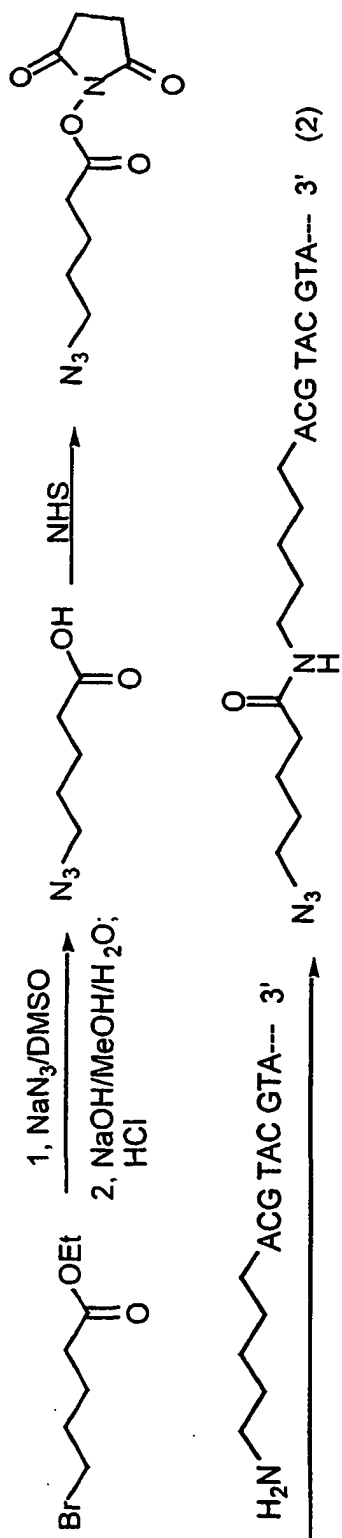


FIGURE 6A

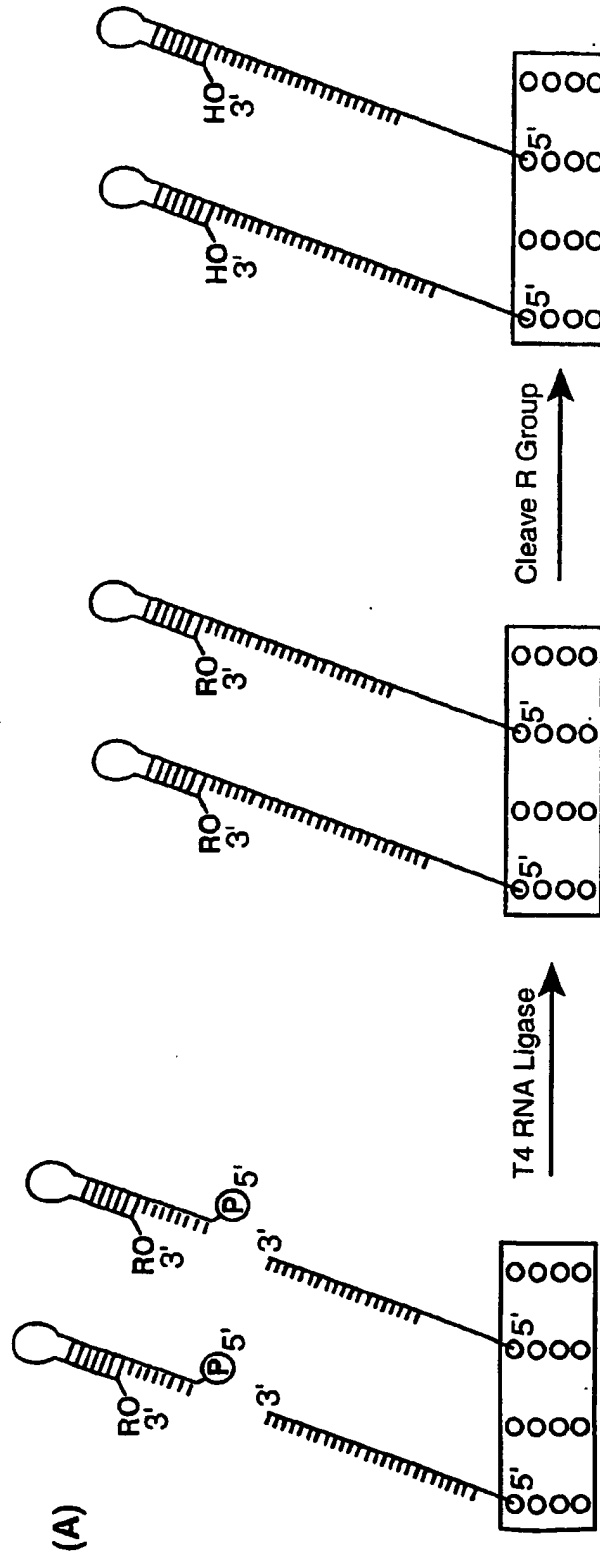


FIGURE 6B

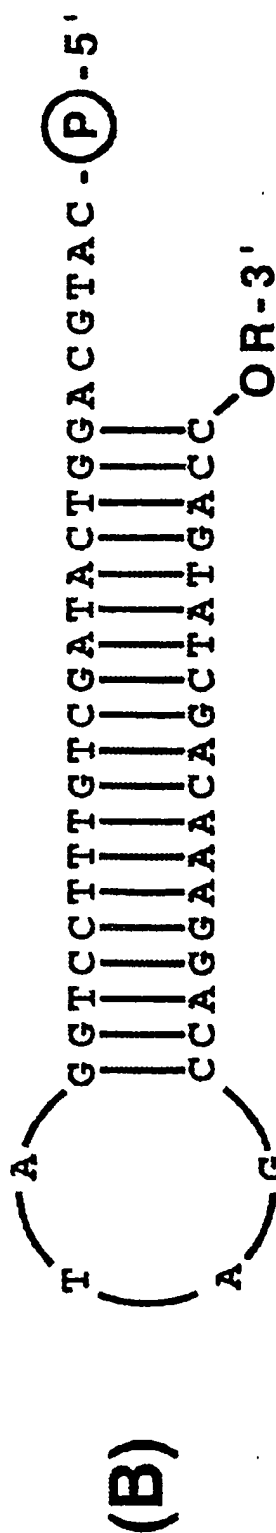
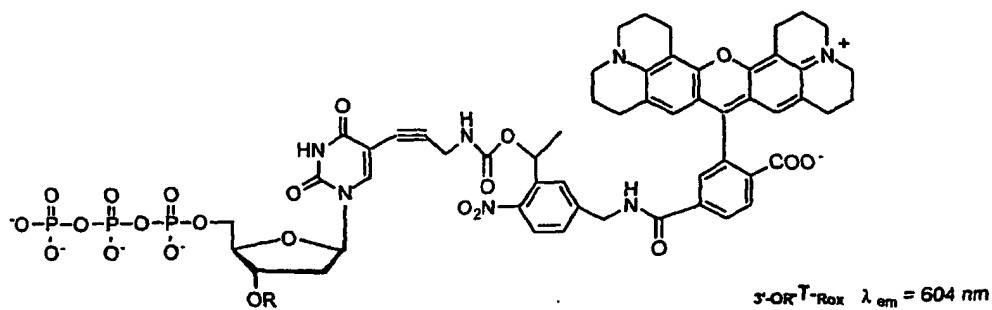
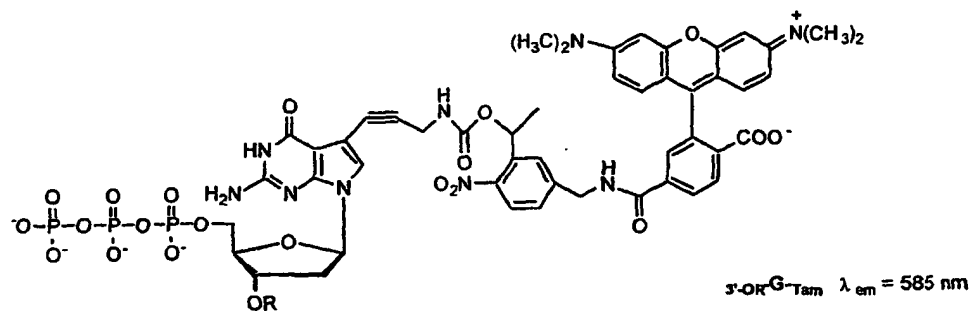
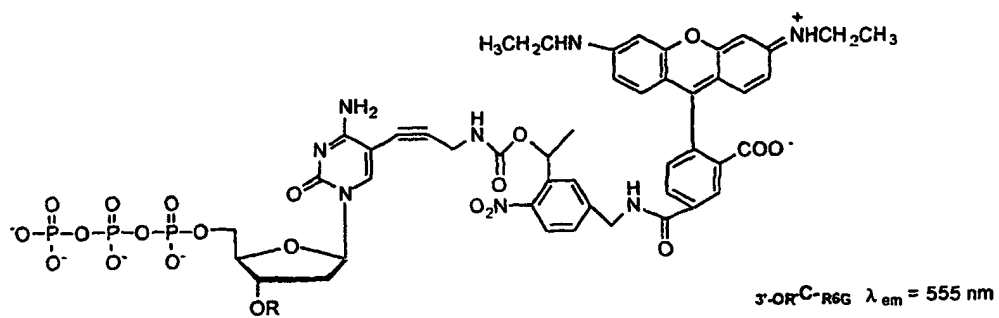
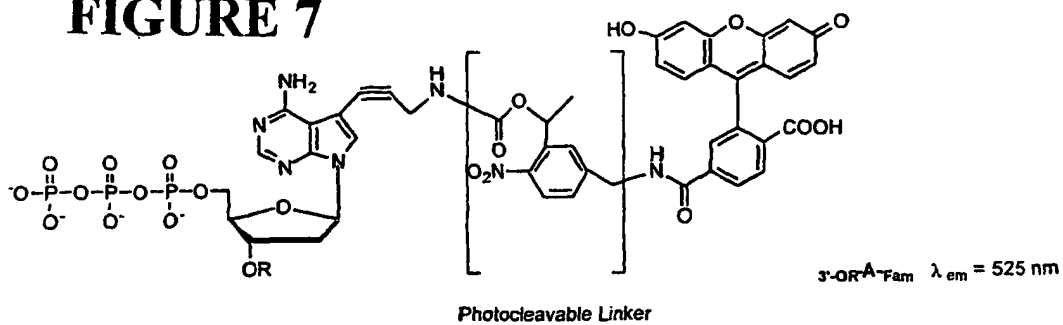


FIGURE 7

R = H, CH_2OCH_3 (MOM) or $\text{CH}_2\text{-CH=CH}_2$ (Allyl)

FIGURE 8

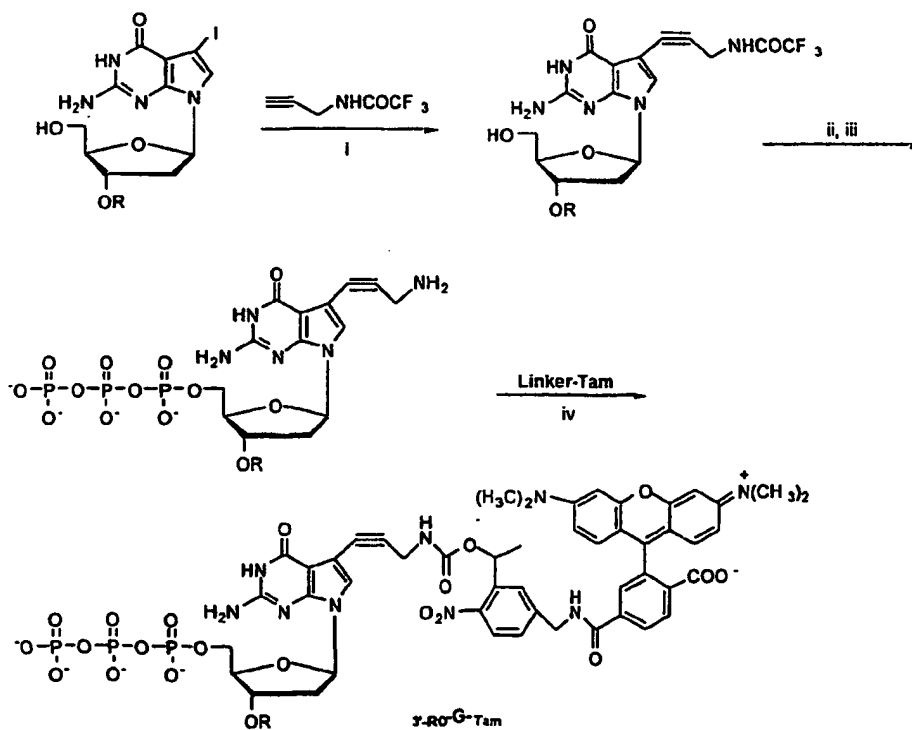
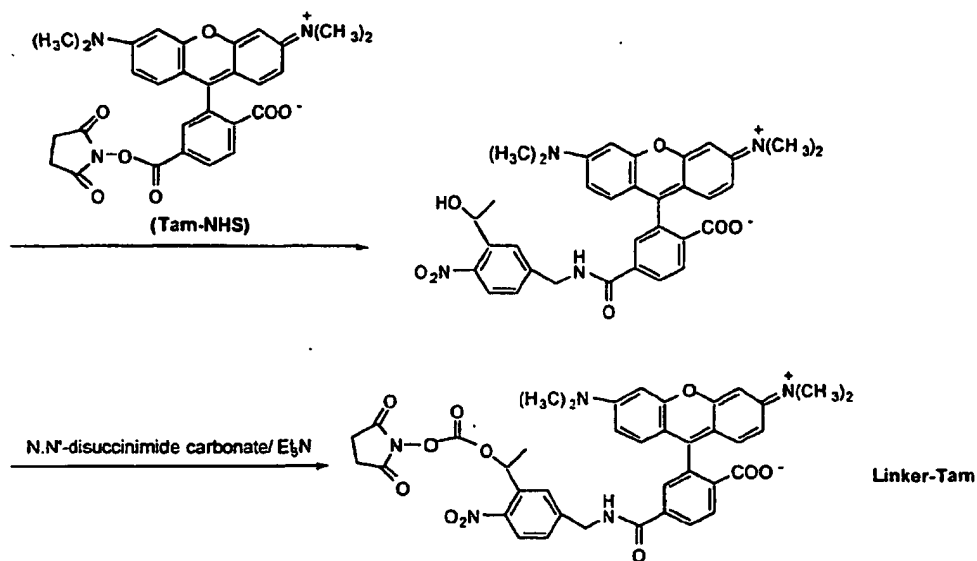
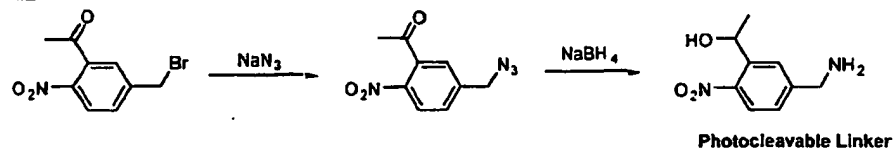


FIGURE 9

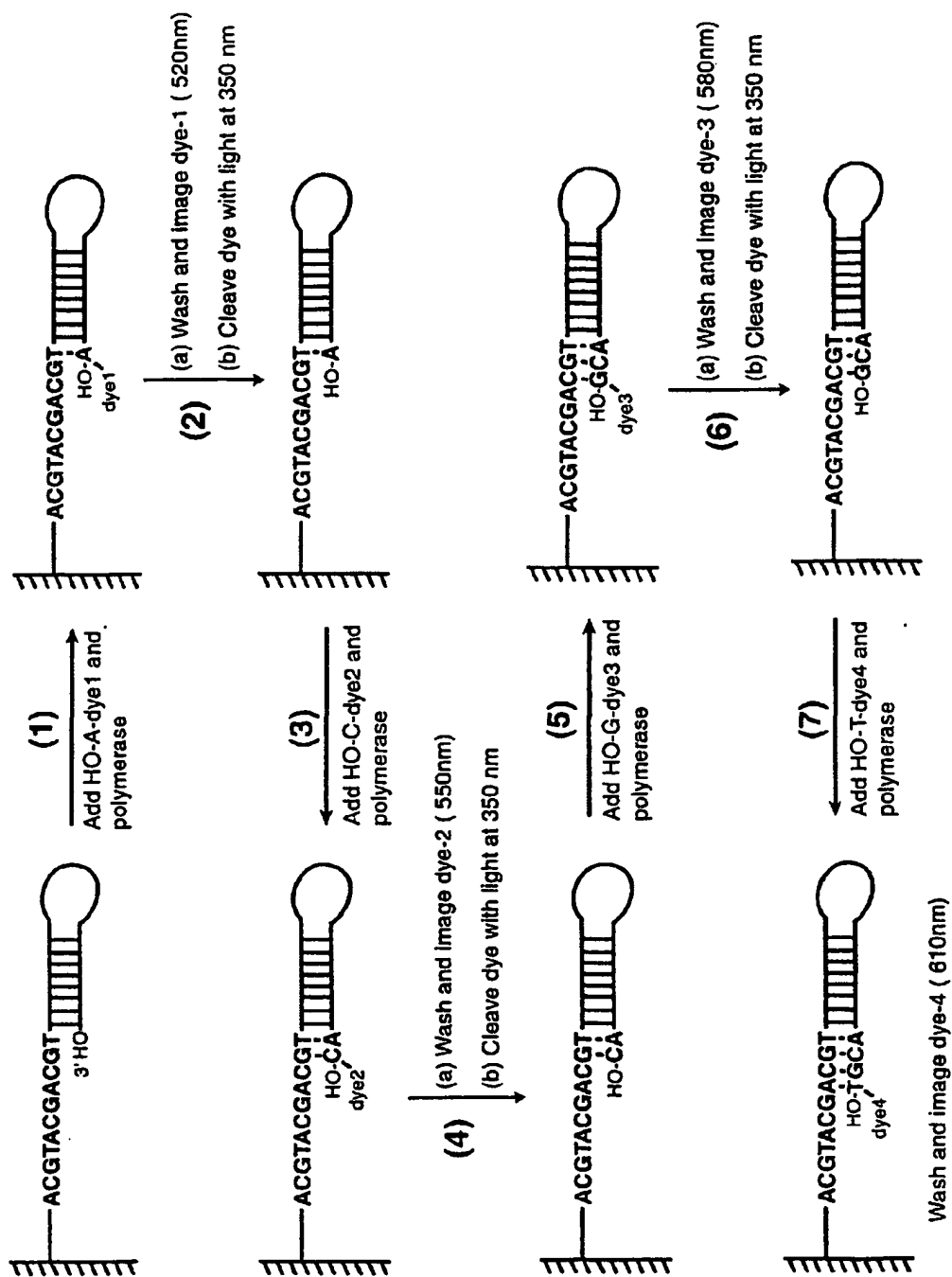
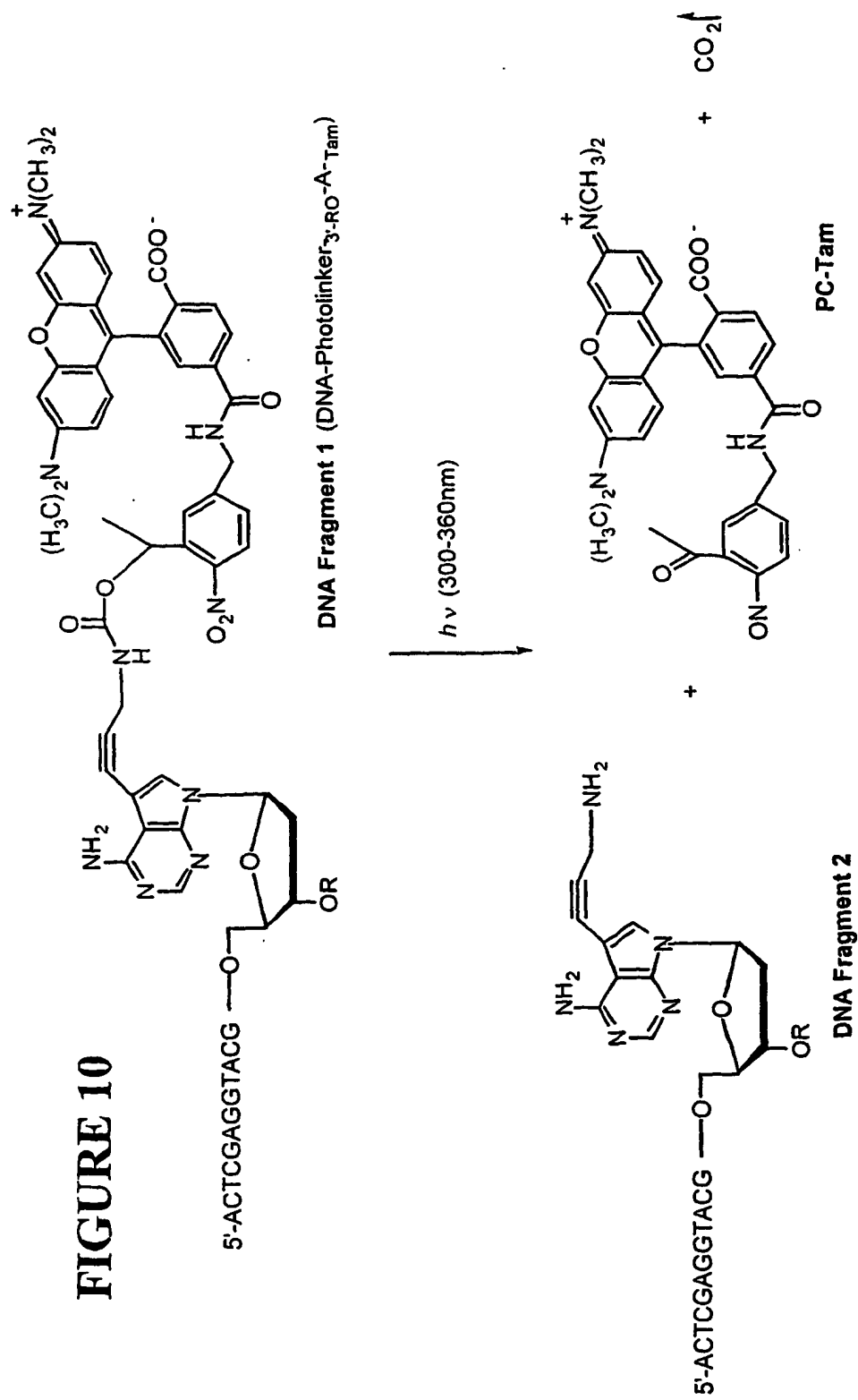
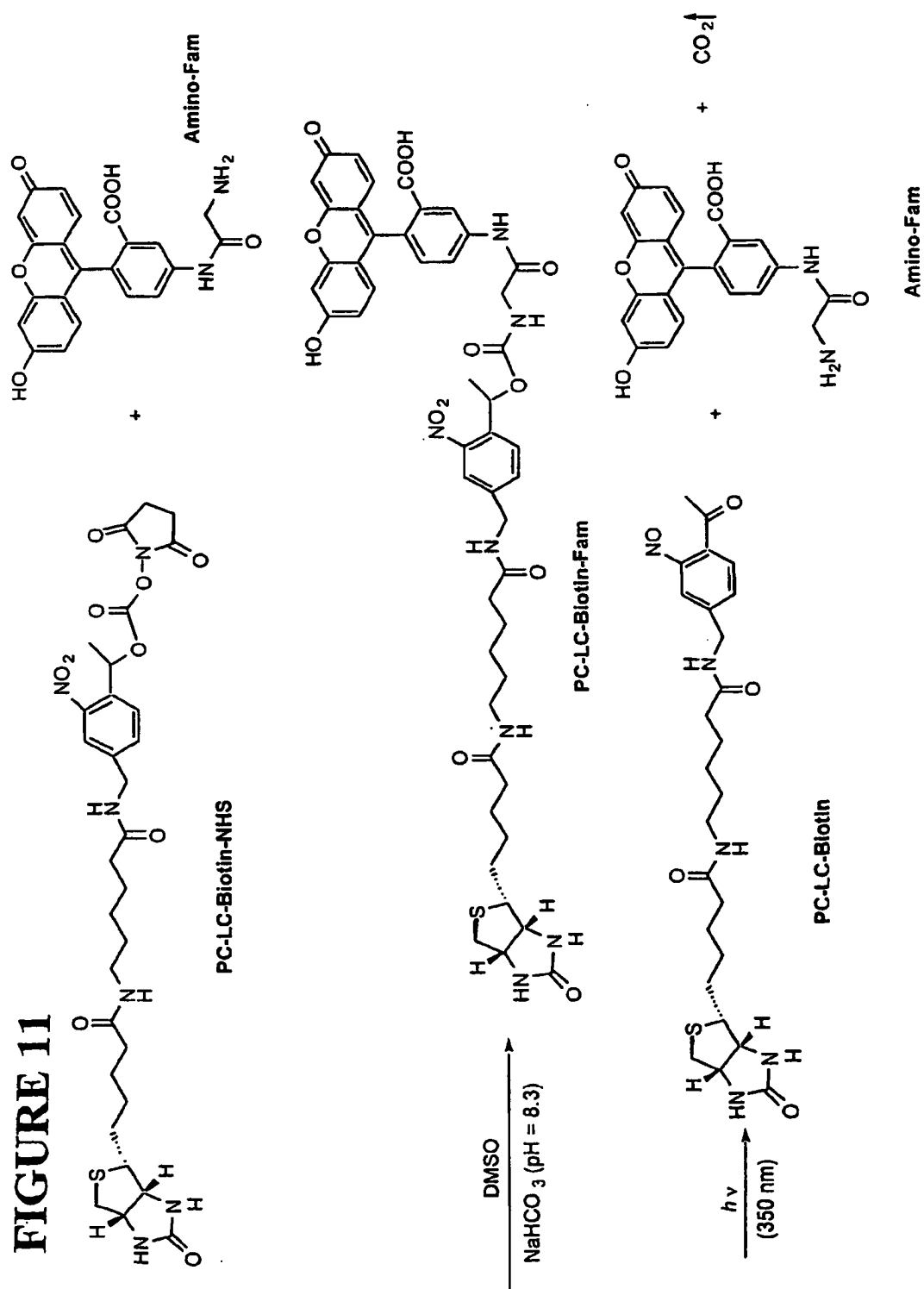


FIGURE 10



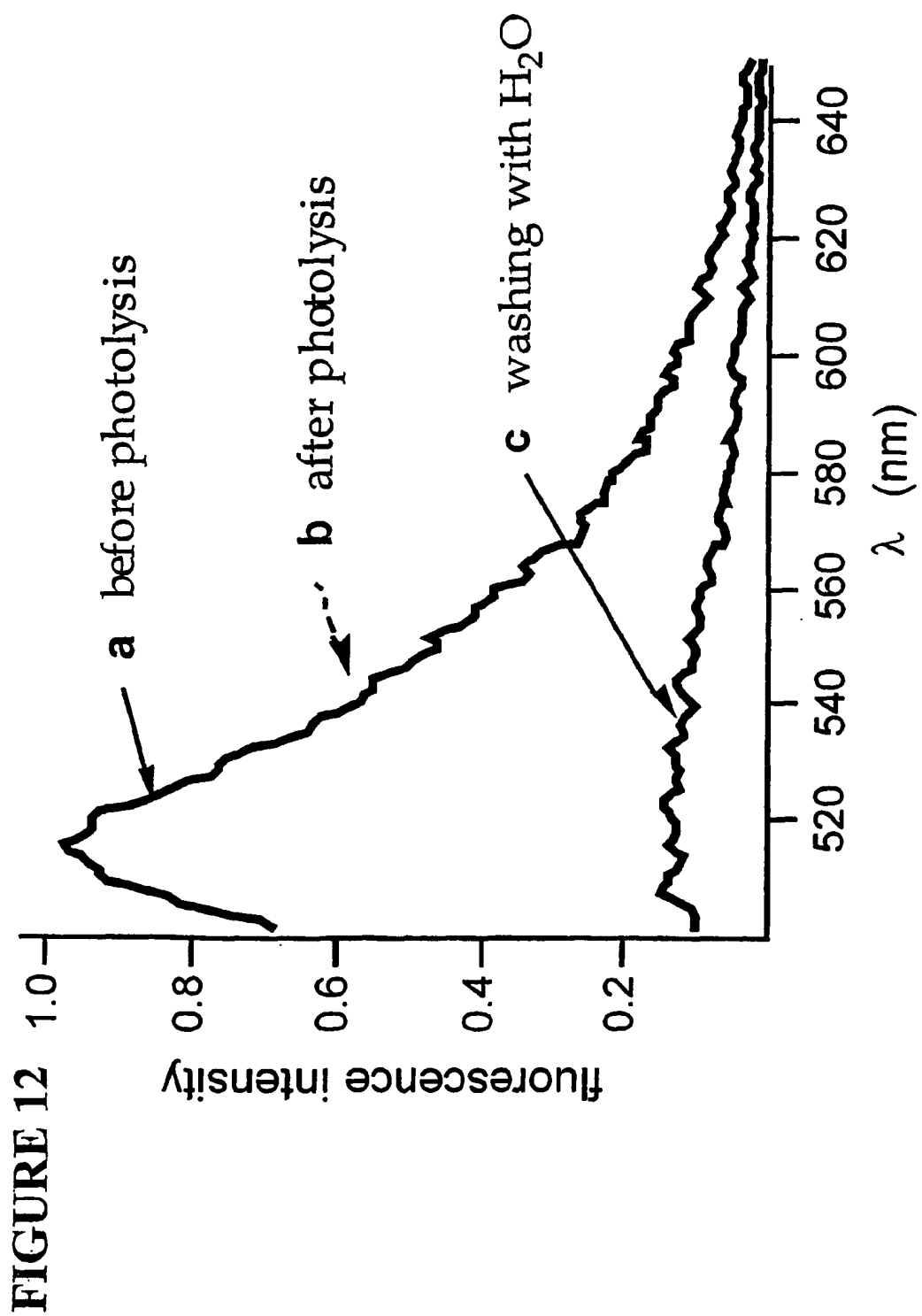


FIGURE 13A

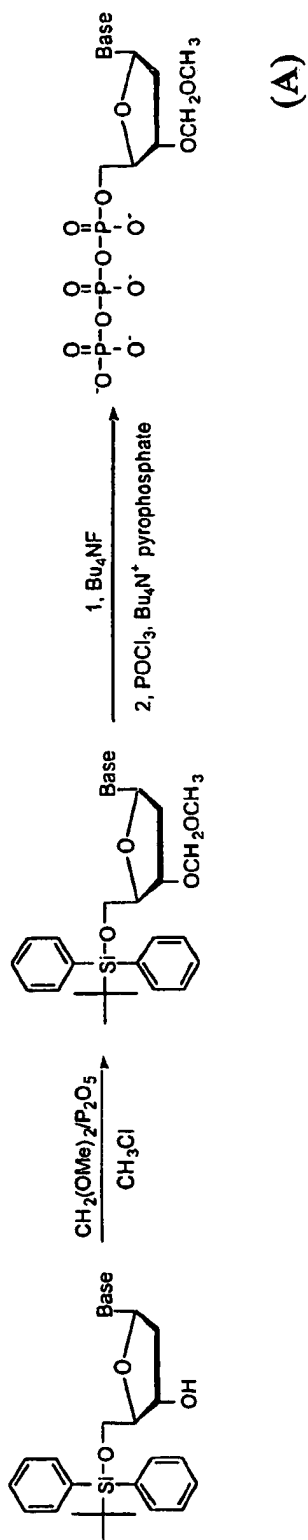
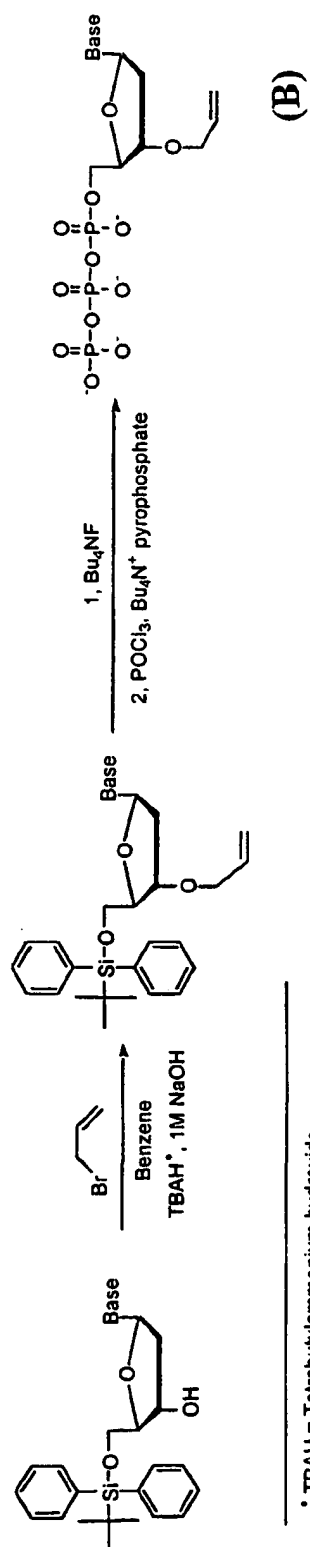
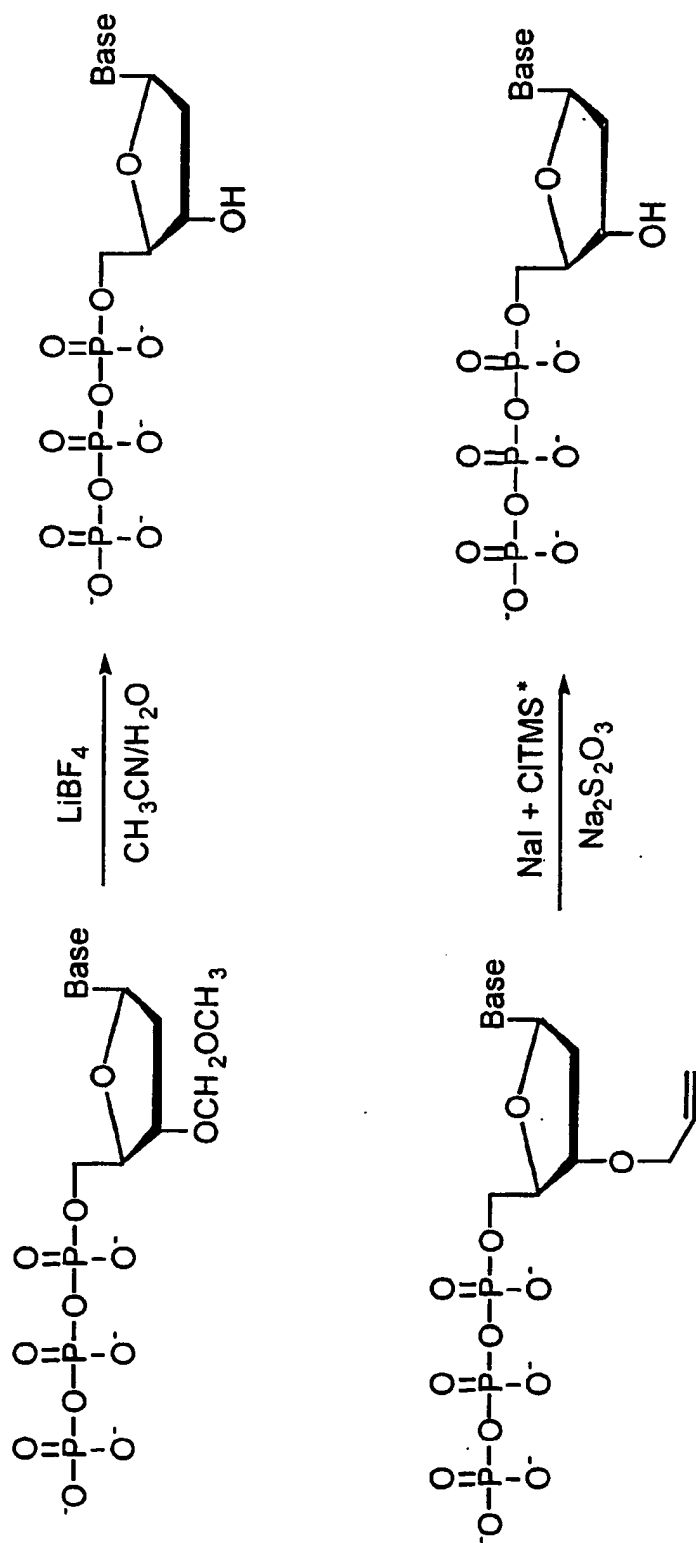


FIGURE 13B



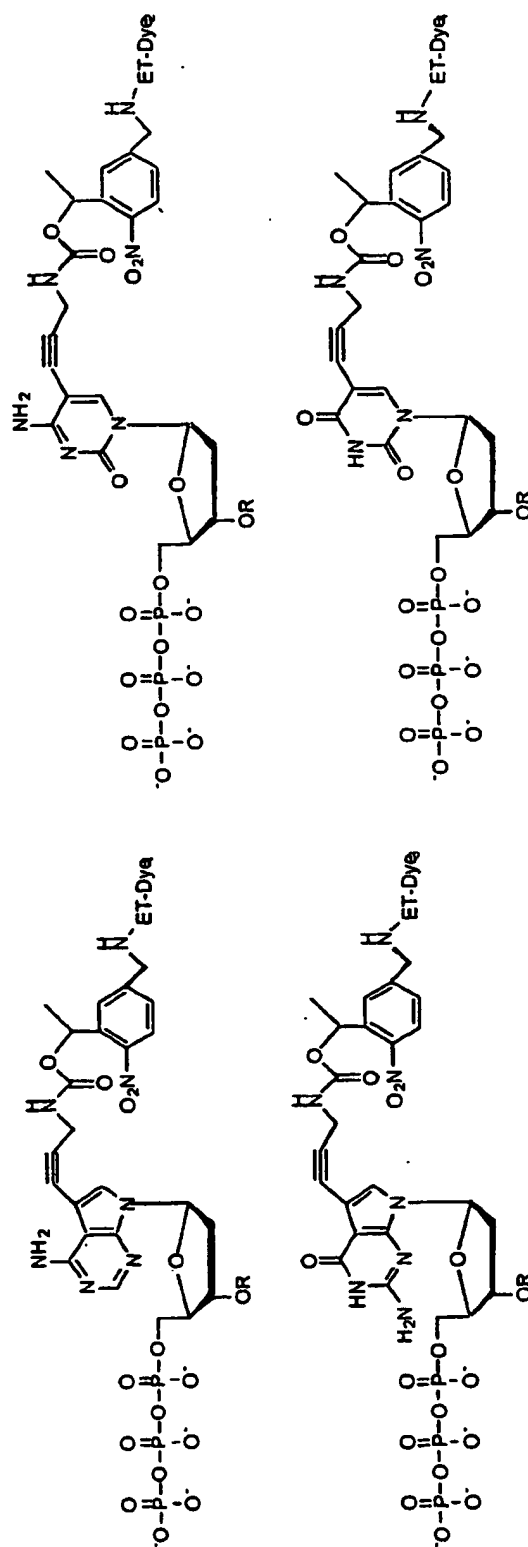
* TBAH = Tetrabutylammonium hydroxide

FIGURE 14



*CITMS = chlorotrimethylsilane

FIGURE 15A



R = H, CH₂OCH₃ (MOM) or CH₂-CH=CH₂ (Allyl)

FIGURE 15B

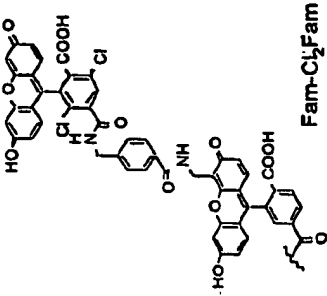
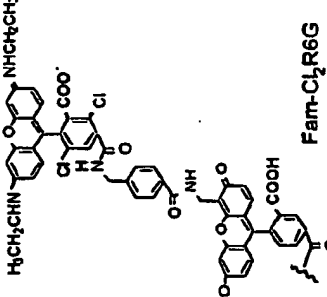
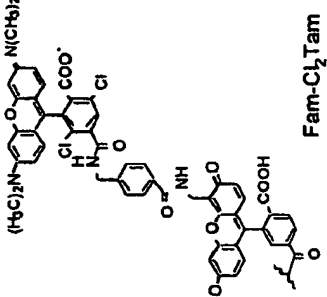
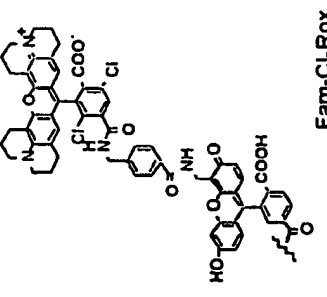
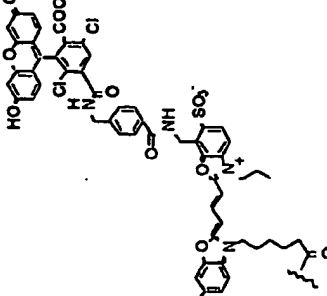
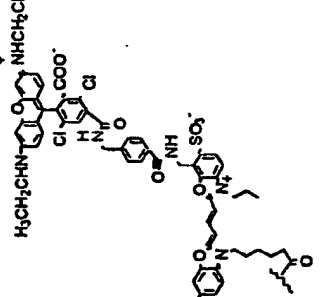
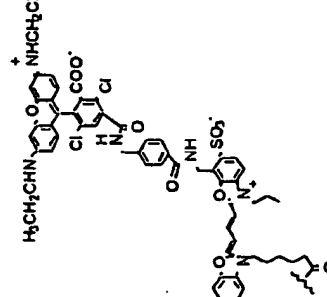
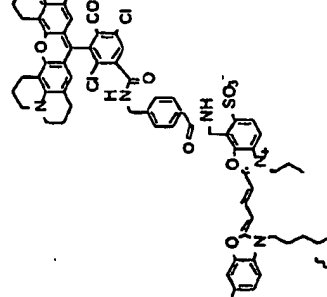
ET Dye ₁ $\lambda_{em} = 530 \text{ nm}$	ET Dye ₂ $\lambda_{em} = 560 \text{ nm}$	ET Dye ₃ $\lambda_{em} = 590 \text{ nm}$	ET Dye ₄ $\lambda_{em} = 620 \text{ nm}$
 <p>Fam-Cl₂Fam</p>	 <p>Fam-Cl₂R6G</p>	 <p>Fam-Cl₂Tam</p>	 <p>Fam-Cl₂Rox</p>
 <p>Cy2-Cl₂Fam</p>	 <p>Cy2-Cl₂R6G</p>	 <p>Cy2-Cl₂Tam</p>	 <p>Cy2-Cl₂Rox</p>

FIGURE 16

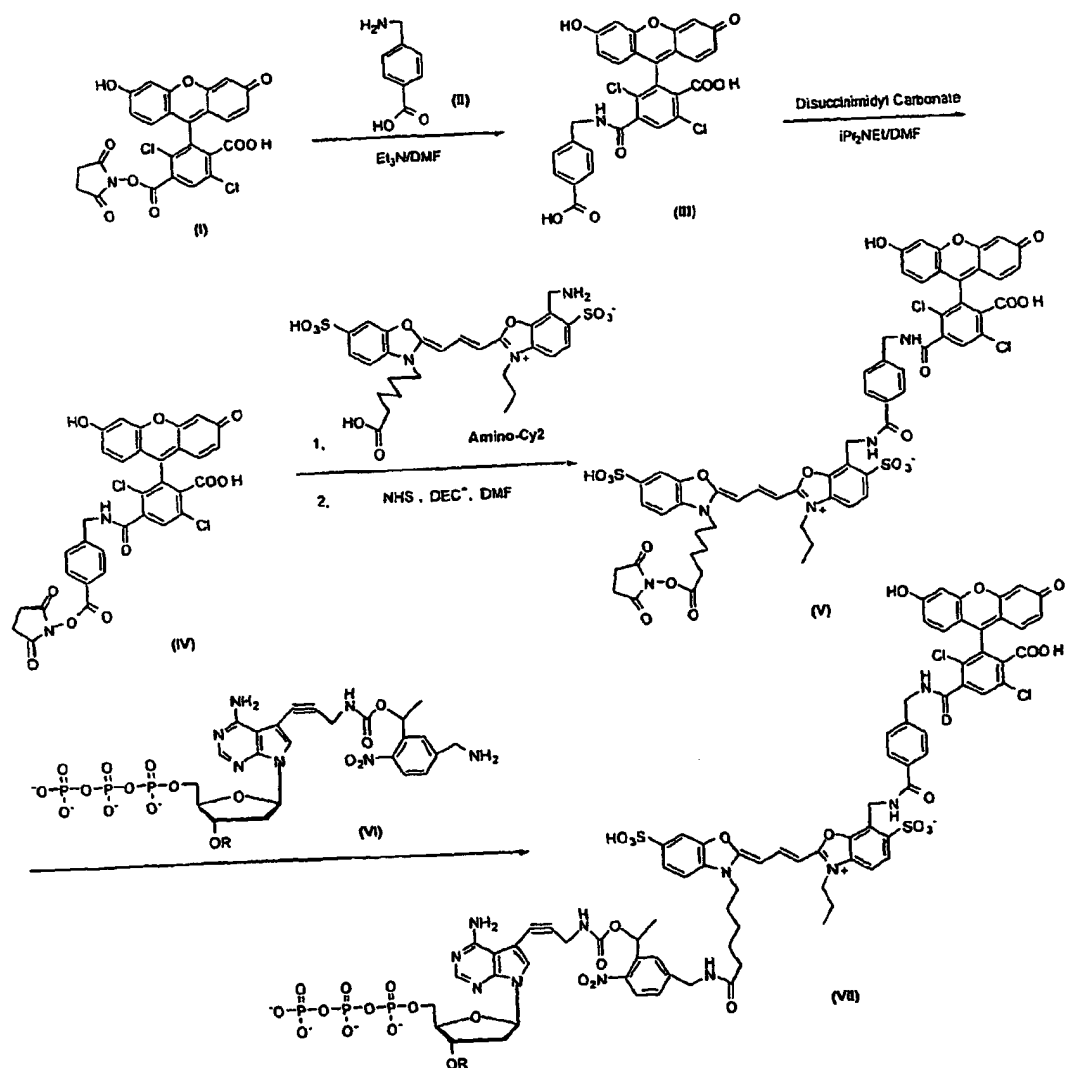


FIGURE 17

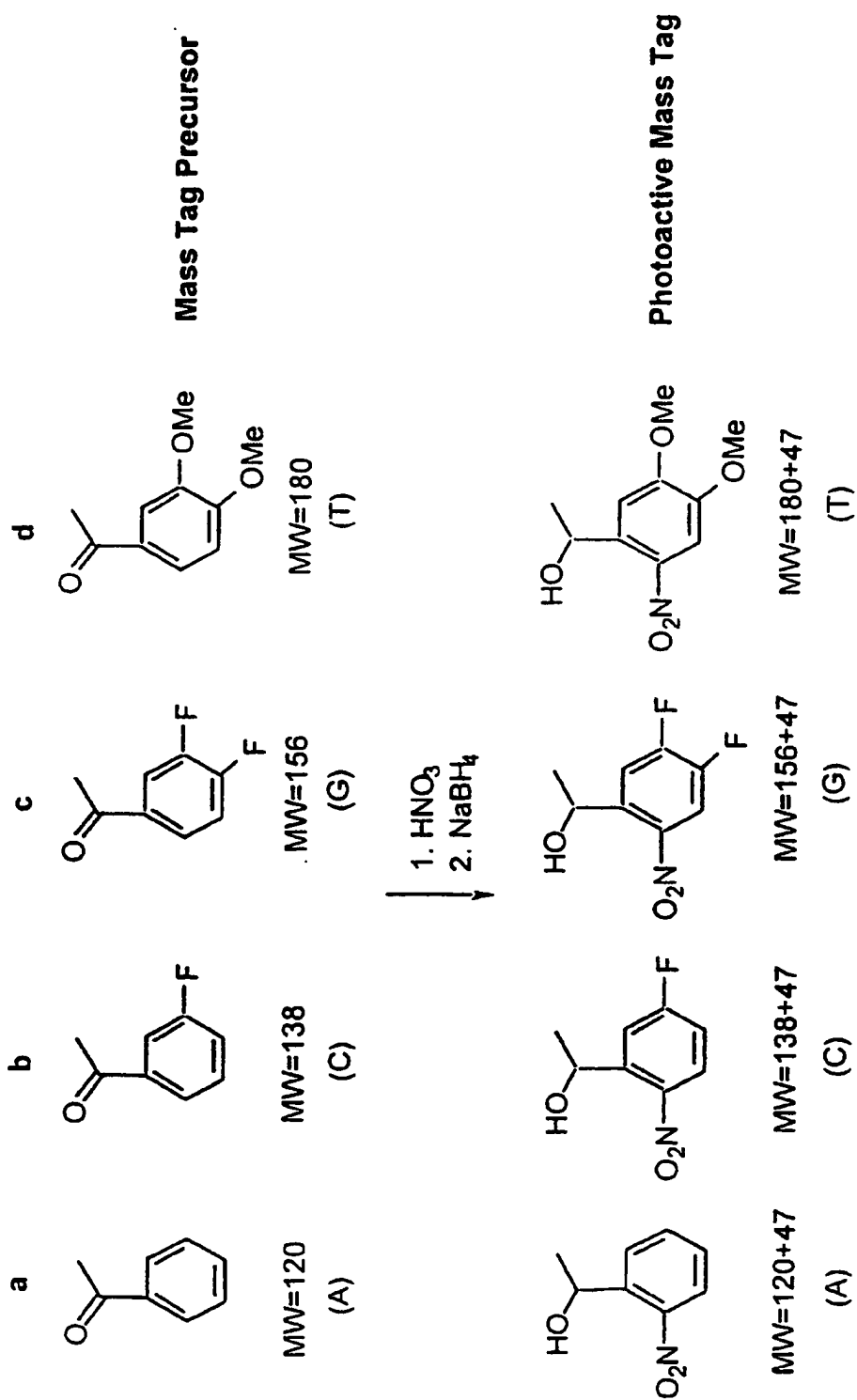


FIGURE 18

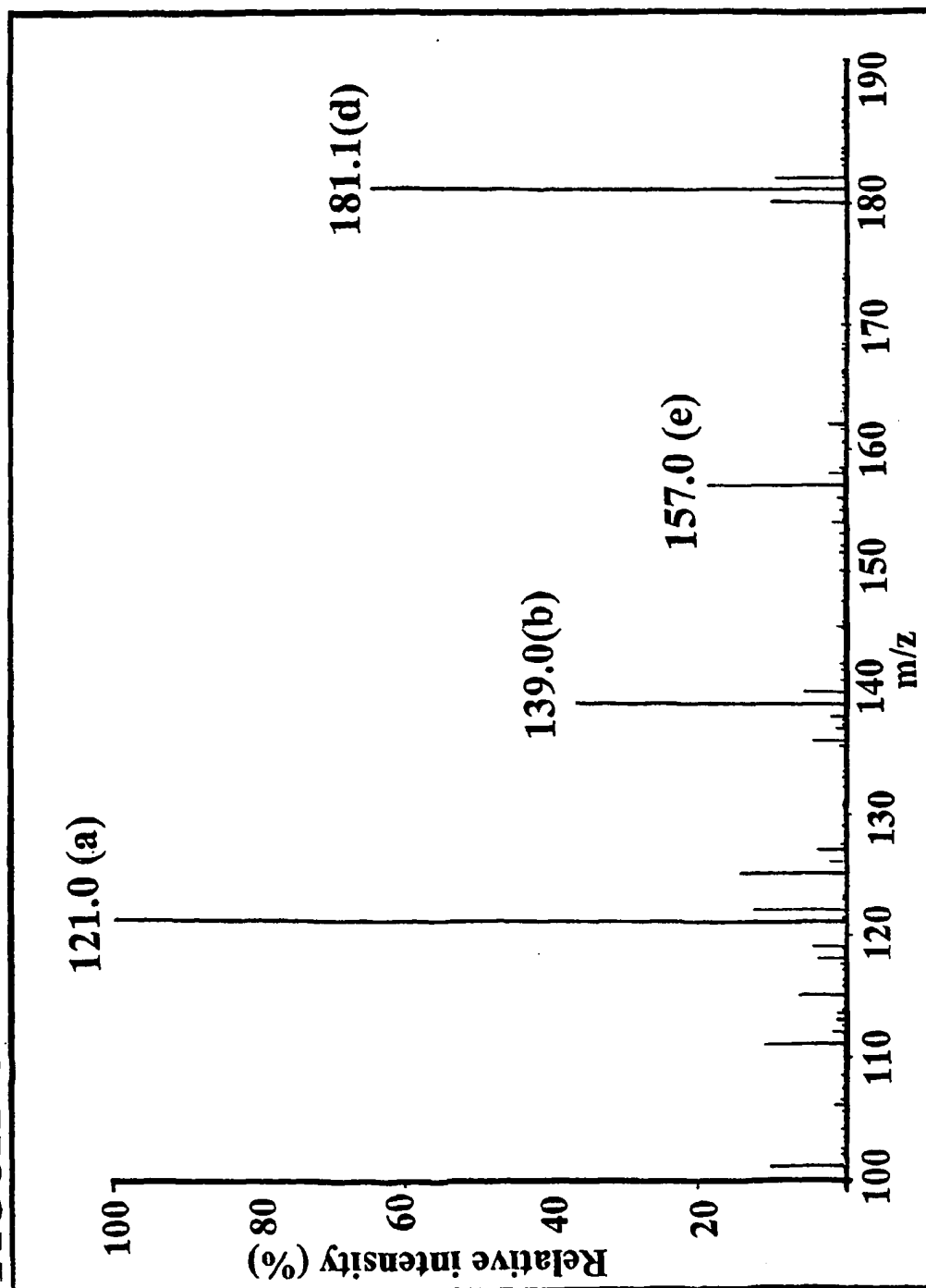
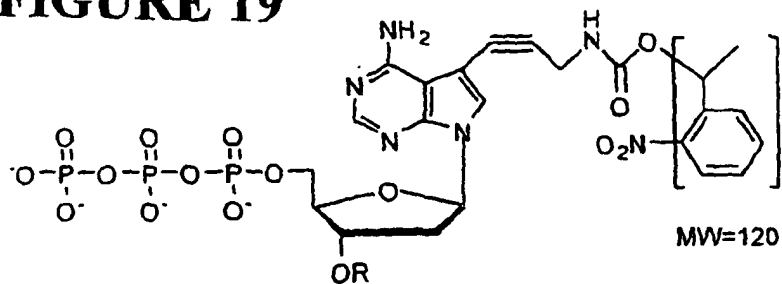
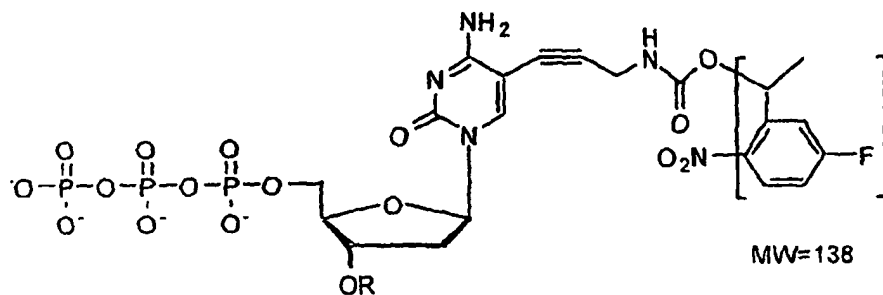
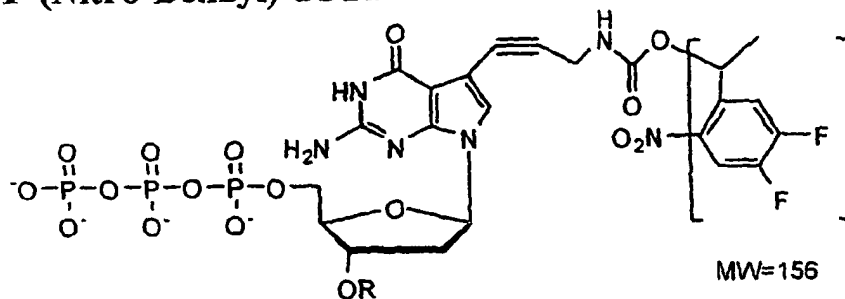
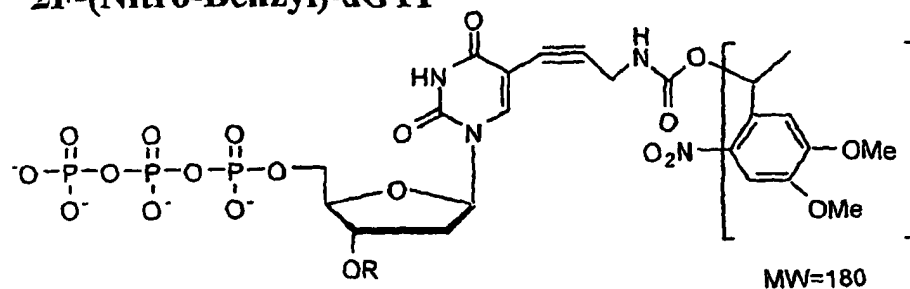


FIGURE 19**(Nitro-Benzyl)-dATP****F-(Nitro-Benzyl)-dCTP****2F-(Nitro-Benzyl)-dGTP****2(MeO)-(Nitro-Benzyl)-dTTP**

R = H, MOM or Allyl

FIGURE 20

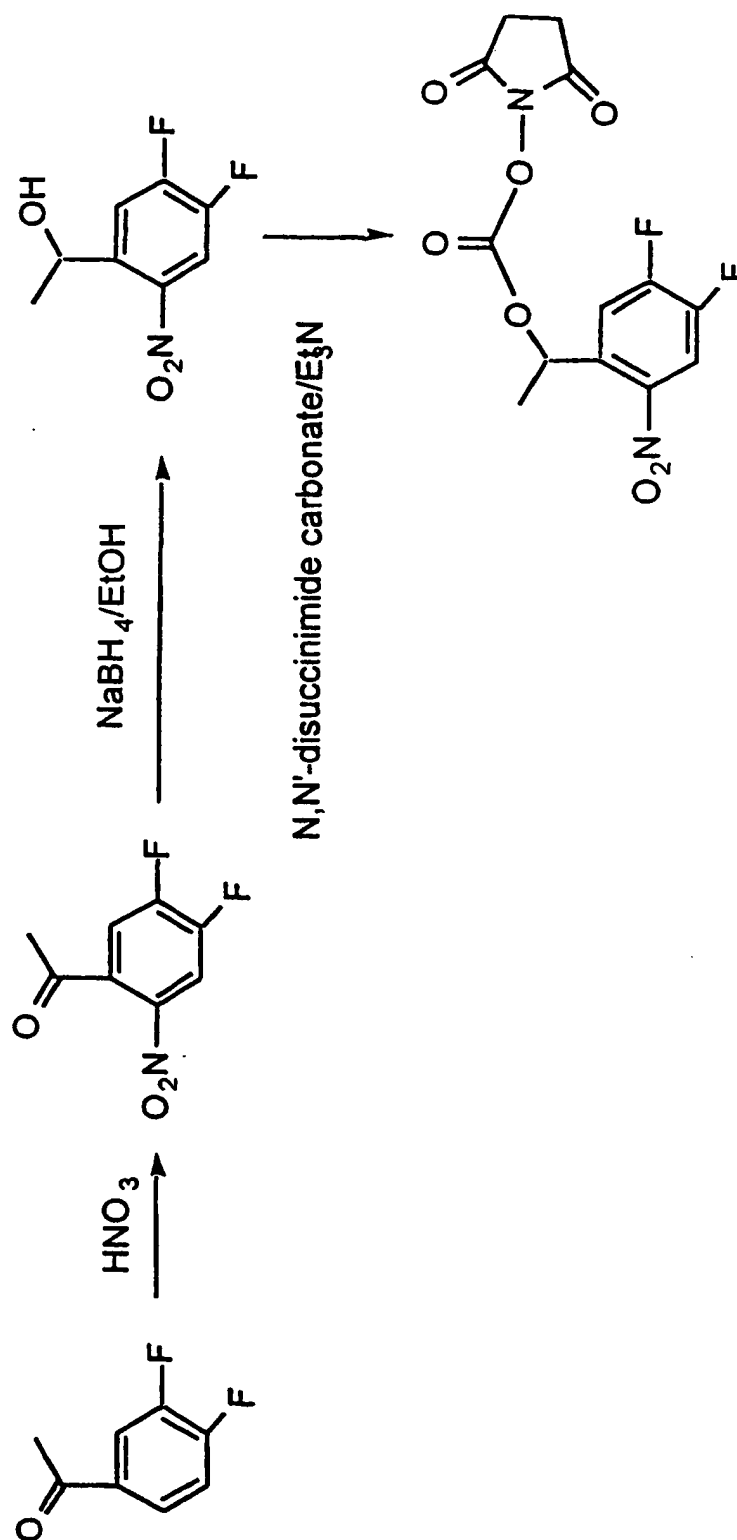


FIGURE 21

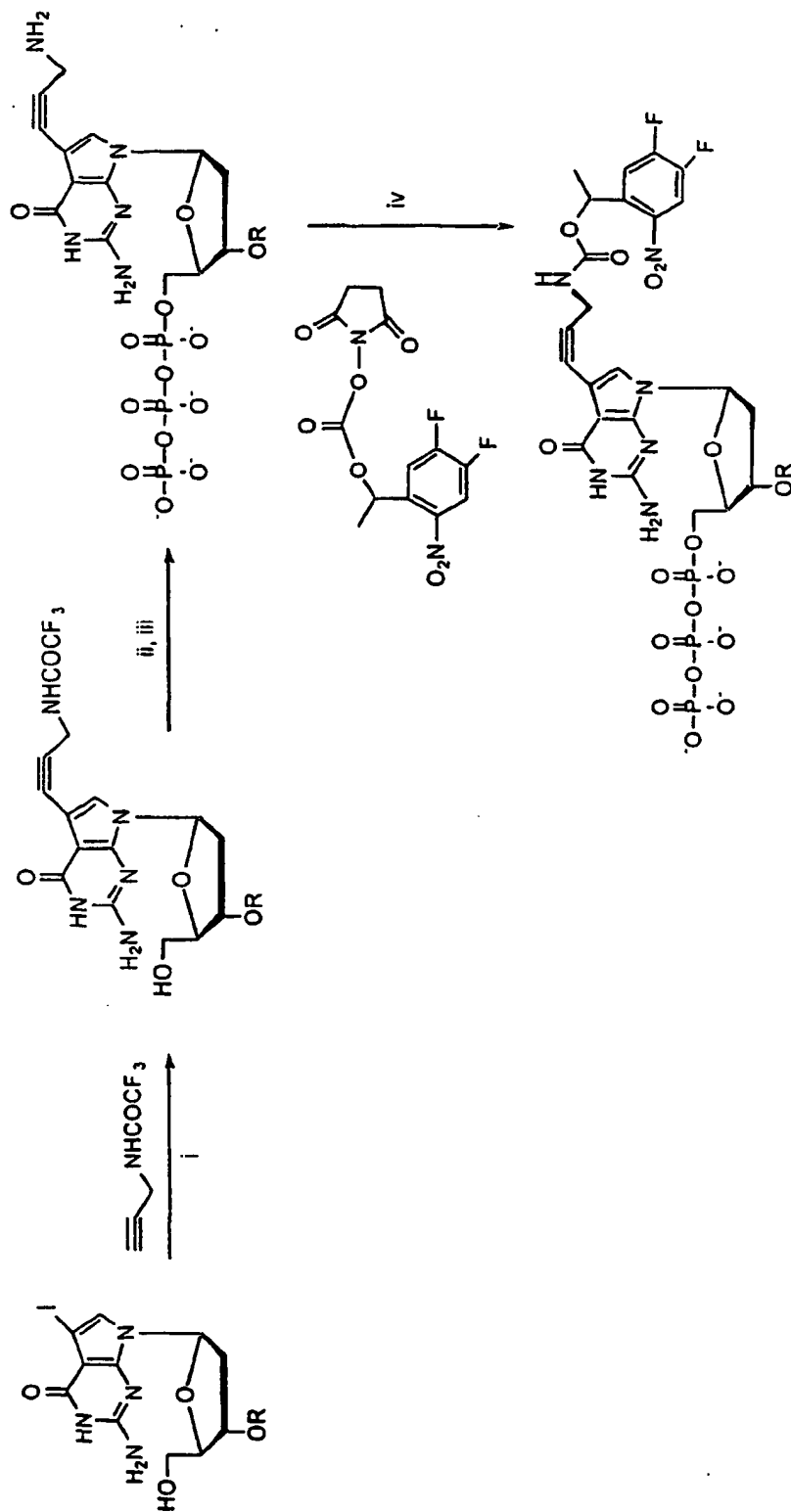


FIGURE 22

